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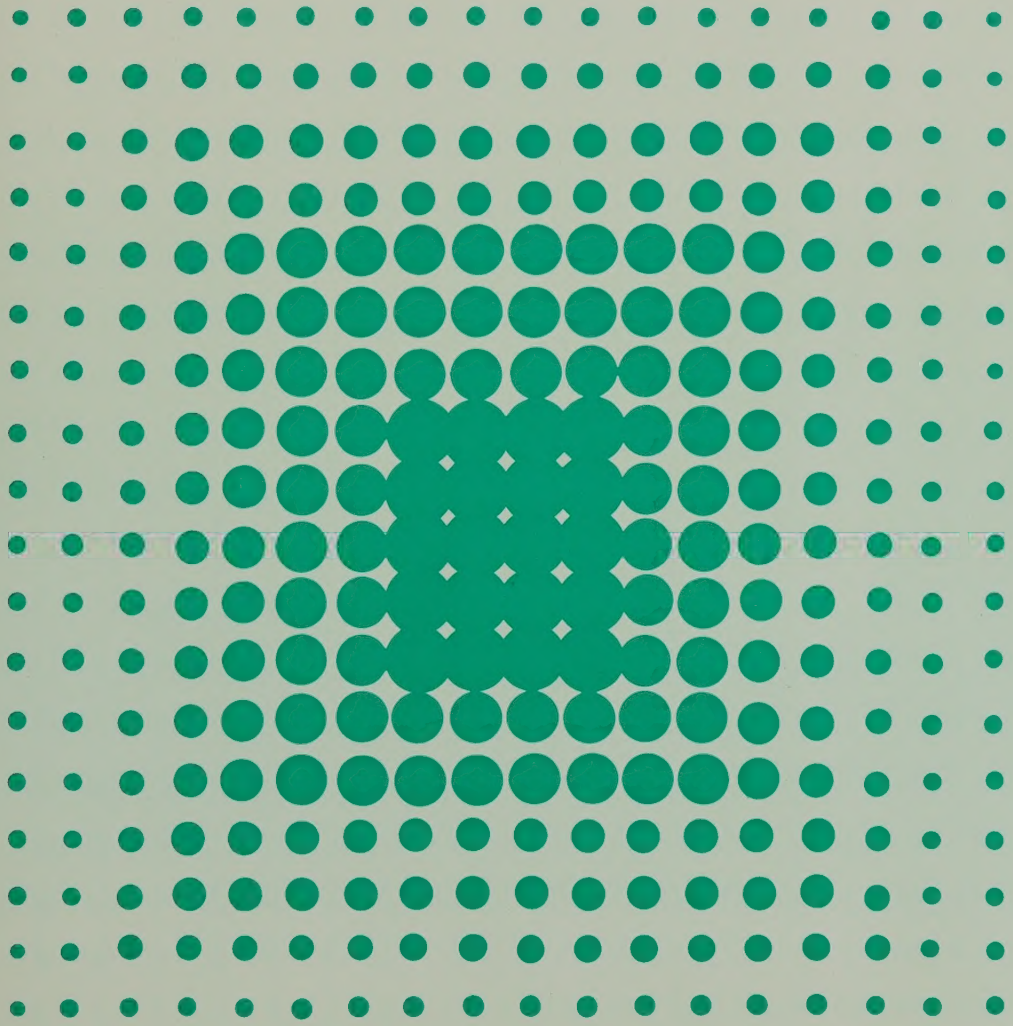

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ORGANIC LINERS FOR THE SEALING OF EARTHEN RESERVOIRS

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Abstract

Soil laboratory columns are used to test the sealing efficiency of organic liners made of various combinations of straw and either beeflot manure or broiler litter. In tests conducted in triplicate each liner was placed at the centre of a 0·6 m core of loam and submerged for 83 days under 3·25 m of low total solids (TS) wastewater. The liquid and contaminant seepage-rate for each liner was monitored throughout the experiment. The liner made of 85% beeflot manure and 15% straw, on a dry matter basis, gave the lowest seepage rate of 19×10^{-9} *m* s^{-1}. *This same liner limited nitrogen seepage rates well below 0·6 g* m^{-2} d^{-1}, *which is the limit required by most North American Environmental Authorities. Extrapolation of the seepage rates, which still decreased after 83 days, indicated that the best liner could reach sealing levels of* 1×10^{-9} *m* s^{-1} *after 128 days.*

Key words: Liners, wastewater, low total solids, gleization.

INTRODUCTION

Earthen structures for the storage of wastewater are economical and environmentally safe if they are built according to specific design criteria. One of these criteria requires that the wastewater contains more than 4% TS (total solids) to initiate a physical sealing process. Low total solids problems can be resolved through the impermeabilization of the soil by enhanced gleization. This technique consists of burying a layer of organic matter at a depth of 0·15–0·30 m under the soil surface to be submerged. This organic layer brings about a microbial reduction process, thereby destroying the structure and reducing the permeability of the soil. An experiment was conducted to test the sealing ability of enhanced gleization through the use of organic liners made of various mixtures of straw and either beeflot manure or broiler litter.

The sealing of soils by wastewater depends mostly on a physical process whereby the solid particles contained in the liquid deposit themselves at the entrance to, and within, the soil pores. This physical sealing process can be enhanced by microbes producing gums and by biochemical reactions destroying the soil pores (Barrington *et al.*, 1987; Rowsell *et al.*, 1985; Davis *et al.*, 1973).

Consequently, wastewater will effectively seal a soil as long as it contains at least 4% TS (Barrington *et al.*, 1990) and the soil has a fine particle-size distribution. High proportions of coarse particles (sands and gravels) lead to larger soil pores and leaching, rather than trapping, of the wastewater solid particles. To ensure physical clogging, Barrington and Broughton (1988) have sugggested minimum soil clay-particle contents of 5 and 15% for manures produced by ruminants and monogastric animals, respectively. Hills (1976) found that clogging requires a soil with at least 8% clay particle content and with a limited particle size over 0·5 mm. Otherwise, physical sealing may not limit seepage rates under the required level of 1×10^{-9} m s^{-1} and the earthen reservoir can contaminate the surrounding groundwater.

Enhanced gleization is a technique which has been used to seal soils exposed to wastewater of low TS (Nicholaichuk, 1978). This process requires a water-logged soil with a minimum organic matter content. Under such conditions, specific anaerobic microbes reduce the medium and solubilize important soil aggregation cements such as magnesium and iron compounds. As a result, the soil loses its structure, its permeability and its large pores (Russell, 1988; Mirtskhulava *et al.*, 1972).

Enhanced gleization has been used to seal earthen irrigation channels as well as reservoirs (Mirtskhulava *et al.*, 1972). The technique requires the burial of an organic layer of 3–4 kg m^{-2} of dry matter some 0·15–0·30 m below the soil surface. Once submerged and under ambient temperatures ranging between 10 and 30°C, a plastic soil layer develops to a depth of 30–50 mm after 4–8 weeks, 150–200 mm after 1 year, and 300–350 mm after 3–4 years. To effectively induce gleization Mirtskhulava *et al.*

(1972) have recommended an organic liner rich in cellulose, such as that made up of grasses, straw and potato beet tops, stalks and leaves.

Enhanced gleization has been tested in the laboratory for the storage of wastewater and water. Tollner *et al.* (1983) used crop residues such as cotton-gin trash and ground corn-cobs to build organic liners to seal sandy loam columns exposed to 1·5% TS of swine manure. Seepage rates as low as 4×10^{-7} m s^{-1} were obtained after 30 days. By submerging in water soils of 40% clay particle content containing a liner of either straw or old manure Nicholaichuk (1978) obtained seepage rates as low as 8×10^{-8} m s^{-1} after 60 days.

Gleization was found to be both longer lasting and as effective as Na dispersion in sealing soil (Nicholaichuk, 1978). Sodium is leached by divalent cations normally dissolved in water in greater concentration. In order to maintain this dispersion effect, Na must be added to the storage water on a regular basis at a concentration giving a sodium absorption ratio (SAR) over 10 (McNeal, 1974; Nicholaichuk, 1978).

Enhanced gleization does not guarantee low seepage rates when the soil is exposed to freezing/thawing and wetting/drying cycles (McConkey *et al.*, 1990*a*, *b*). A ten-fold increase in seepage rate has been observed with such cycles, even with the addition of sodium carbonate. Therefore, all earthen structures sealed by enhanced gleization should never be emptied, in order to protect them against the adverse effects of drying and freezing.

An organic liner can enhance the process of gleization, if (Sorensen, 1982; Knowles, 1982):

— the C/N ratio is balanced at 20;
— it contains cellulose of variable decomposition rate;
— it possesses a low permeability because of its well-distributed particle size.

METHODS

A loam was used to build the soil cores within the experimental column (Tables 1 and 2). The experimental soil was obtained from the C horizon (1·0 m depth) of a site found in the Canadian Prairies in the region of Brooks, Alberta. This loam had an organic matter content of 1·7% and a clay particle content of 24%. The high level of organic matter is typical of some soils in the Canadian Prairies, where the groundwater table remains within 1·0–2·0 m of the soil surface even during the summer months. The soil had a moisture content of 19%, by weight, when it was packed manually into the experimental columns.

Straw, solid beeflot manure and broiler litter were the organic materials tested as liners (Tables 1 and 2). The straw and manure combination were designed to give a variable C/N, a range of hydro-

Table 1. Chemical composition of the experimental material

Property	Soil	Experimental material Organic matter			Wastewater
		Straw	Beeflot manure	Poultry manure	
Dry matter, %	80·6 (1·15)	92	36	74	—
T.S., mg l^{-1}					233 (137·9)
pH	8·2 (0·21)	—	—	—	8·1 (0·00)
C.E.C., meq kg^{-1}	147 (3·4)	—	—	—	2·44 (0·00)
E.C., ms cm^{-1}	16·9 (1·35)	—	—	—	2·44 (0·00)
O.M., % d.b.	1·7 (0·10)	97·5 (1·27)	98·4 (8·95)	87·7 (9·92)	—
TKN g kg^{-1} d.b.	0·88 (0·150)	7·00 (0·20)	34·00 (4·20)	43·00 (460)	—
mg l^{-1}	—	—	—	—	210 (4·24)
NH_4-N mg kg^{-1} d.b.	4·2 (1·19)	300	9000	5400	—
mg l^{-1}	—	—	—	—	198 (4·2)
NO_3-N mg kg^{-1} d.b.	1·2 (0·35)	—	13·5	76·0	—
mg l^{-1}	—	—	—	—	0·55 (0·0707)
C/N	10·5	77·4	16·3	11·2	—
Ca + Mg, mg kg^{-1} d.b.	112 (20·2)	—	—	—	58·5 (2·34)
mg l^{-1}	—	—	—	—	58·5 (2·34)
Na mg kg^{-1} d.b.	121 (13·3)	—	—	—	114 (0·82)
mg l^{-1}	—	—	—	—	114 (0·82)
K mg kg^{-1} d.b.	2·15 (0·374)	—	—	—	—
SAR	2·2	—	—	—	3·0

Note: The organic matter content was converted to carbon by dividing by 1·8. The value in parenthesis is the standard deviation.

Table 2. Physical properties of the experimental material

Physical property	Experimental material			
	Soil	Broiler litter	Beeflot manure	Straw
1. Particle size, %				
<2 μm	34 (1·71)	—	—	—
<45 μm	54 (2·67)	23·6 (2·11)	20·1 (2·88)	5·5 (2·10)
<125 μm	— (2·77)	40·2 (6·89)	38·9 (2·10)	5·5
<500 μm	100 (5·28)	61·0 (11·40)	56·3 (10·32)	6·0 (2·81)
<1000 μm	—	95·4 (18·09)	98·5 (12·39)	94·0 (4·22)
Bulk density after Proctor compaction				
— wet, g ml^{-1}	2·04 (0·061)			
— dry, g ml^{-1}	1·66 (0·102)			

Note: The value in parenthesis is the standard deviation.

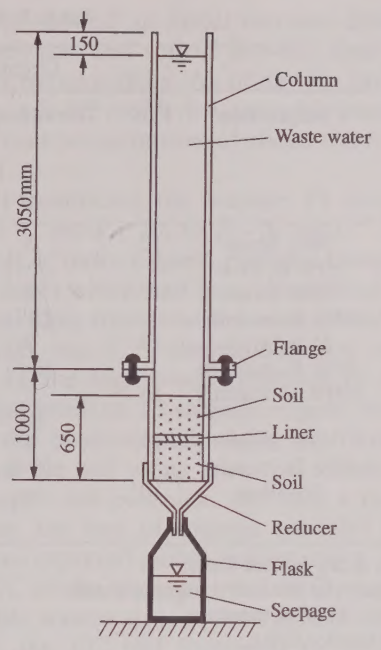

Fig. 1. The experimental column.

carbon decomposition rates and a well-distributed particle size.

Slaughterhouse wastewater was used to test the liners (Table 1). This wastewater had been left to settle before collection and therefore had a TS of 233 mg l^{-1}. This wastewater also contained 210 and 0·55 mg l^{-1} of TN (total nitrogen) and nitrate, respectively. The SAR value of 3 for the wastewater was well below 10, leading to clay particle dispersion.

Experimental columns

The experimental columns were built of two sections of 150 mm inside diameter polyvinyl chloride (PVC) tubing assembled by means of flanges (Fig. 1). The bottom 0·1 m section was designed to hold the soil core and the organic liner, while the top 3·05 m section was held to the bottom section by sealed flanges. The column drainage was collected by means of a reducer and its volume was used to calculate the column's seepage rate. Eighteen columns were built to test five treatments and one control in triplicate.

The experimental columns were set up in a non-insulated building, tested during the period from July to September and exposed to ambient temperatures from −4 to −31°C. In early October, however, the experiment had to be terminated as temperatures consistently dropped below 0°C during the night.

Methodology

The 18 columns tested the following treatments and control, each in triplicate (Table 3):

(1) The sealing performances of four types of liners were compared. The four types of liners were made of 50% straw with 50% beeflot manure (treatment 1), 67% straw with 33% broiler litter (treatment 2), 15% straw with 85% beeflot manure (treatment 3) and 100% beeflot manure (treatment 4), all on a dry-matter basis. To maintain equal amounts of TS among treatments, the wastewater level of each column was maintained by refilling the column with its own seepage.

(2) The field performance of the organic liner was tested by repeating treatment 3 as treatment 5, except for the refilling of these columns with more wastewater, rather than with their own seepage liquids.

(3) The performance of the four types of organic liners was tested against a control: the soil itself without any liner. It should be noted that the soil used had a relatively high level of organic matter and this can also bring about a certain level of gleization.

The soil was manually packed into the bottom section of the columns to obtain a relatively high soil hydraulic conductivity (k) with respect to that induced by gleization. The soil core density was obtained by measuring the total depth of soil and liner placed in each column and by weighing all the column bottom sections once they were filled (Table 3). The variable density of the soil cores did not affect the experimental results as the initial seepage rate, reflecting k, was some 1000 times higher than that induced by gleization.

In order not to disturb the liner, a metal plate covered the soil cores when the columns were filled with 3·25 m of wastewater. The seepage rates of all

Table 3. Description of experimental column

Treatment	Organic liner						Soil core			
	Composition[a]	C/N[b]	Thickness, cm	Weight, kg	Density		Bottom depth cm	Top depth cm	Density	
					wet g ml^{-1}	humid g ml^{-1}			wet g ml^{-1}	dry g ml^{-1}
1.	50% Straw	22	8	0·108	0·075	0·062	32	30	1·87	1·50
	50% B. litter		(1·5)	—	(0·0131)	(0·0105)	(1·5)	(0·6)	(0·101)	(0·084)
2.	67% Straw	42	7	0·154	0·126	0·084	32	30	1·84	1·48
	33% B. manure		(2·3)	—	(0·0335)	(0·0225)	(1·0)	(0·6)	(0·044)	(0·038)
3.	15% Straw	19	3	0·207	0·437	0·190	32	31	1·82	1·51
	85% B. manure		(0·8)	—	(0·1318)	(0·0573)	(0)	(0·6)	(0·0513)	(0·0458)
4.	100% B. manure	16	1	0·225	1·274	0·510	32	31	1·72	1·38
			(0)		(0)	(0)	(1·15)	(0·6)	(0·083)	(0·068)
5.	15% Straw	19	4	0·207	0·273	0·119	32	30	1·81	1·46
	85% B. manure		(0·6)		(0·034)	(0·0144)	(0)	(1·5)	(0·023)	(0·026)
6.	No liner	10[a]	—	—	—	—	31	30	1·74	1·31
							(2·3)	(3·2)	(0·095)	(0·049)

Notes: [a]On a dry matter basis.
[b]The C/N ratio of the soil's organic matter.
B. manure — beeflot manure.
B. litter — broiler litter.
The value in parenthesis is the standard deviation.

the columns were monitored by measuring the volumes of liquid collected by the reducers at intervals over the experimental period. The seepage rate was calculated as

$$I = V/8{\cdot}64 \times 10^{-2}/A/\mathrm{d}t \qquad (1)$$

where I=seepage rate (m s^{-1}); V=volume of liquid collected under the column over a period of time dt (ml); A=cross-sectional area of the soil core (m^2); dt=time during which V was collected (days).

For example, a seepage volume of 15 ml collected over 7 days gave a seepage rate of 1×10^{-8} m s^{-1}.

Every two weeks, a sample of the seepage was analyzed for pH, TKN, NH_4-N, NO_3-N, TS and EC (electrical conductivity) using standard methods (APHA, 1990).

The treatment seepage rates were statistically analyzed at a confidence level of 95%, using the method of analysis of variance (AOV) as described by Steel and Torrie (1980). A block-treatment system was used, with the blocks representing the time of sampling. The new Duncan's multiple range test was used to determine which treatment demonstrated a significantly different seepage rate.

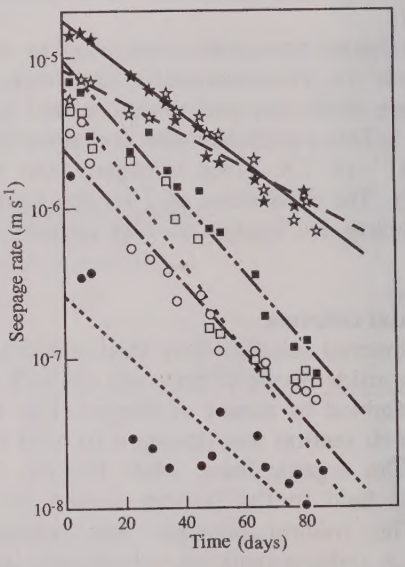

Fig. 2. Seepage rate as a function of time. Treatment 1 ———★; treatment 2 — —☆; treatment 3 —·—○; treatment 4 —··—■; treatment 5 - - -●; treatment 6 — —□.

RESULTS AND DISCUSSION

Seepage rates

Treatment 3 (85% beeflot manure and 15% straw) gave the lowest seepage rate of 19×10^{-9} m s^{-1} after 83 days (Fig. 2; Table 4). Treatments 4 and 6 (100% beeflot manure liner and the control) were significantly better than treatments 1 and 2 (50% straw and 50% broiler litter; 67% straw and 33% beeflot manure) which still demonstrated a seepage rate of 1×10^{-6} m s^{-1} at the end of the experiment. No significant difference was found to exist between treatments 1 and 2, or between treatments 4 and 6.

Treatment 5 performed much better than treatment 3, hence it can be concluded that the addition of new wastewater to a submerged soil can further enhance gleization. These results also demonstrate the importance of using the seepage liquids to refill columns when comparing the sealing performance of various treatments. Otherwise, those treatments with a higher seepage rate receive an additional amount of wastewater TS and perform as well as the others.

The seepage rates of all treatments were still declining after 83 days of experimentation. Linear regression of the logarithmic value of the seepage rate was therefore used to extrapolate the results. Table 4 lists the regression equation obtained for each treatment, as illustrated in Fig. 2. Thus, the equation indicated that treatment 5 could have reached the environmentally acceptable seepage rate of 1×10^{-9} m s^{-1} after 128 days.

Quality of the seepage

For most column seepage, the high initial contamination concentration diminished with time (Figs 3 and 4).

Table 4. Statistical comparison of liner infiltration rates

Treatment	Regression equation	r	Sealing time[a]
1	$I=10^{-4.81-0.015t}$	−0·98	279
2	$I=10^{-5.81-0.011t}$	−0·93	290
3	$I=10^{-5.60-0.023t}$	−0·96	147
4	$I=10^{-5.05-0.023t}$	−0·98	172
5	$I=10^{-6.56-0.019t}$	−0·79	128
6	$I=10^{-5.17-0.026t}$	−0·95	147

Note: I — seepage rate, m s^{-1}.
t — time, days.
r — correlation coefficient.
[a]To reach a seepage rate of 1×10^{-9} m s^{-1}.

The pH showed an initial increase above that of the wastewater and soil of 8·0–8·2, thus indicating active microbial activity. As of day 55, the pH dropped from 8·5–8·7 to 7·9–8·3, probably because of the cool ambient temperatures of about 1°C from day 45 to day 51.

For all treatments, the seepage TS dropped from 200 mg l^{-1}, on day 10, to 25–70 mg l^{-1} on day 40, as a result of more organic particles being caught in the soil pores which had been diminished in size by gleization. Only treatment 4 demonstrated a high TS above 200 mg l^{-1} throughout the experiment because of the high biodegradability of its organic liner. High levels of TS seepage suggest the degradation of the physical seal which contributes to the sealing of the soil along with the effects of gleization. Despite soil pore size, and thus k reduction by gleization, the loss of organic particles signified a lower than expected global sealing effect.

The EC of the seepage was initially equivalent to that of the wastewater, of 2·44 mmhos cm^{-1}. After 20 days, the EC had increased to 10–20 mmhos cm^{-1} because of the displacement of salts in the soil by the cations of the seepage and the release of salts from the manure. Treatment 4 demonstrated the highest levels of EC, of 20 mmhos cm^{-1} after 20 days because of the higher content of its liner manure.

The TKN and NH_4-N of the seepage were initially of the order of 200 and 125 mg l^{-1}, but dropped

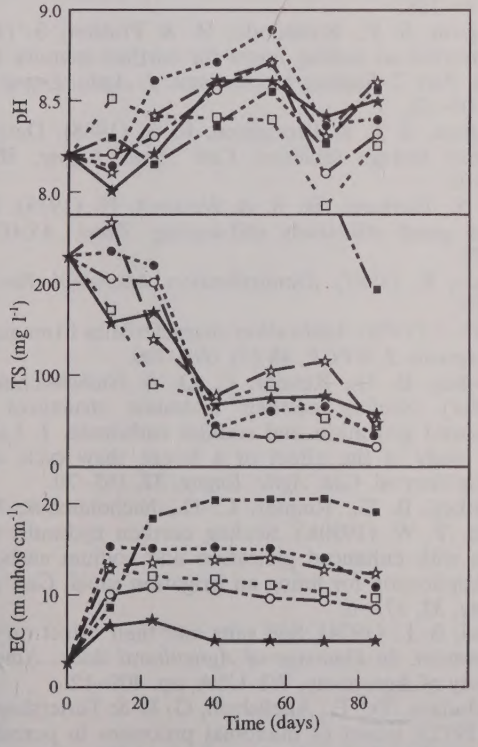

Fig. 3. Seepage pH, TS and EC as a function of time. Treatment 1 ———★; treatment 2 — —☆; treatment 3 —·—○; treatment 4 —··—■; treatment 5 - - -●; treatment 6 — —□.

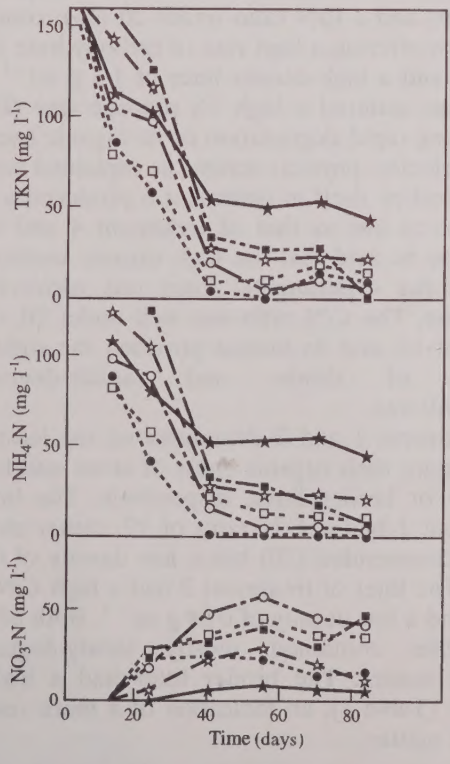

Fig. 4. Seepage N as a function of time. Treatment 1 ———★; treatment 2 — —☆; treatment 3 —·—○; treatment 4 —··—■; treatment 5 - - -●; treatment 6 — —□.

below 50 and 25 mg l^{-1}, respectively, after day 40. Nitrates increased from 0 to 50 mg l^{-1}, from day 0 to day 40. Nevertheless, TN (total nitrogen) at day 40 was much lower than that of the wastewater, at 210 mg l^{-1}. All treatments attained the required TN seepage rate of 0·6 g m^{-2} day^{-1}, after 83 days of testing.

Quality of the organic liner

The quality of the organic liner was found to influence the extent of gleization. The four types of organic liner, along with the control, offered variable C/N ratios, variable hydrocarbon bio-degradability and variable liner density or permeability. The following liner properties gave the best sealing performance:

a C/N ratio under 20:
a combination of straw and manure to give a combination of slowly- and rapidly-biodegradable carbohydrates.
a high organic liner density obtained from a well-distributed particle size or from a combination of straw and manure.

Accordingly, the liner of treatment 3 produced the lowest seepage rate because it had a C/N ratio below 20 (19); a straw to beeflot manure ratio of 15/85, giving a balanced rate of carbohydrate degradation, and a dry density of 0·3–0·4 g ml^{-1}. This liner therefore provided the best conditions for induced gleization.

The liner of treatment 4, the second-best-performing liner, had a C/N ratio under 20 (16); contained no straw, offering a high rate of carbohydrate degradation; and a high-density liner of 1·3 g ml^{-1}. This treatment suffered a high TS seepage rate (Fig. 4) suggesting rapid degradation of its organic liner and a less effective physical sealing, as explained earlier.

The soil by itself in treatment 6 produced a seepage rate as low as that of treatment 4 and it can therefore be said that the high organic matter content of the experimental loam was conducive to gleization. The C/N ratio was well under 20, of the order of 10, and its humus provided the right proportion of slowly- and rapidly-degradable carbohydrates.

Treatments 1 and 2 demonstrated the least sealing, despite their organic liners of straw and beeflot manure or broiler litter, respectively. The liner of treatment 1 had a C/N ratio of 22, rather close to that recommended (20) but a low density of 0·62 g ml^{-1}. The liner of treatment 2 had a high C/N ratio of 42 and a low density of 0·84 g ml^{-1}. Both of these treatments contained mostly slowly-degradable organic matter. The broiler litter had a high ash content (Table 1), an indication of a more resistant organic matter.

Physical factors affecting sealing

The seepage rates were found to depend on ambient temperatures. Treatment 5, for example, showed an increase in seepage rate from 20 to 45×10^{-9} m s^{-1} between days 45 and 51 as the ambient minimum temperatures dropped from 12 to 1°C and rose again to 8°C.

The high hydraulic head of 5–6 applied to the soil cores may have influenced the performance of the organic liners. Previous laboratory experiments have been conducted with low hydraulic gradient (the depth of liquid and soil core divided by the depth of soil core) of 1·0 or less. A high hydraulic gradient results in higher seepage rates, along with faster leaching of microbes. The microbes may have been leached faster than they could reproduce, especially for treatments 1 and 2, where microbial growth was slowed by the lack of rapidly-degradable carbohydrates.

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EVALUATION OF HYDROLYSIS CONDITIONS OF CELLULOSIC MATERIALS BY *PENICILLIUM* CELLULASE

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Abstract

The conditions of hydrolysis of 'skop' (short fiber waste material from the paper industry) and cellolignin (waste of industrial furfural production) by the cellulase system from Penicillium *sp. in a batch reactor have been evaluated. The optimal conditions were: enzyme (filter paper activity) — 1·7 U/ml, skop — 100 g/l, cellolignin — 150 g/l, pH 4·5, temperature 50°C. Agitation was essential for cellolignin but not essential for skop hydrolysis.*

Studies of fed-batch process of saccharification of both substrates by three cellulase preparations (from Penicillium *sp.,* Trichoderma reesei *and combined preparations of* T. reesei *and* Aspergillus foetidus*) have shown that the highest saccharification activity was demonstrated by* Penicillium *cellulase.* Penicillium *cellulase was able to hydrolyze, during 48 h, three portions of skop or cellolignin with a degree of cellulose conversion to glucose of 68–84% and final glucose concentrations of 93–98 g/l. The use of fed-batch saccharification decreased consumption of enzyme and increased the final glucose concentration compared to batch hydrolysis.*

The role of different factors in decreased rate of skop and cellolignin hydrolysis by Penicillium *cellulase in the course of reaction has been evaluated. The main factors influencing the efficiency of saccharification were decrease of skop and cellolignin reactivity during hydrolysis and inhibition of enzymes by glucose and cellobiose.*

Key words: Cellulase, *Penicillium* sp., enzymatic hydrolysis, cellulosic material.

INTRODUCTION

Previously we reported on the kinetic properties and stability of cellulolytic enzymes produced by *Penicillium* sp. fungi, comparing it to enzymes from *Trichoderma reesei* (Castellanos *et al.*, 1993). Vlasenko *et al.* (1993) investigated the susceptibility of cellulose-containing materials (CCM) representing approximately 30 potential raw materials available in Russia to saccharification by *Penicillium* cellulase. The highest initial rate of glucose formation and degree of substrate conversion were demonstrated with a waste product of the paper industry known as skop and with cellolignin, a waste material of furfural production.

For maximum efficiency of hydrolysis of these substrates it is necessary to optimize enzymatic saccharification conditions and minimize consumption of expensive cellulase preparation. Benefits include reduced glucose production cost and increased probability of making enzymatic hydrolysis profitable.

The most critical property limiting the enzymatic hydrolysis of insoluble cellulose is decreased reaction rate at low degree of substrate conversion (Fan *et al.*, 1987). The main reasons for this are: decrease of substrate susceptibility to enzymes (Lee & Fan, 1983; Sinitsyn *et al.*, 1991), end-product inhibition (Ladisch *et al.*, 1983; Holtzapple *et al.*, 1990; Ramos *et al.*, 1993), thermal inactivation of enzymes (Gusakov *et al.*, 1982), inactivation by stirring (Sinitsyn *et al.*, 1986), reversible inactivation of enzymes adsorbed on cellulose surfaces (Gusakov *et al.*, 1985), and their inactivation on lignin fragments (Ooshima *et al.*, 1990). The contribution of each individual factor in the process of hydrolysis is affected by reaction conditions, such as enzyme to substrate ratio, pH, temperature, stirring (Gusakov *et al.*, 1985), and each factor should be evaluated.

The objectives of this work were to determine the optimal conditions of hydrolysis of skop and cellolignin by cellulase from *Penicillium* sp. We attempt to achieve the most efficient utilization of enzyme by employing periodic substrate additions (fed-batch process). This article evaluates the contribution of different factors affecting the efficiency of hydrolysis.

METHODS

Cellulases

Enzyme preparations from *Penicillium* sp. (freeze-dried culture filtrate) were provided by the Institute of Biochemistry and Physiology of Microorganisms (Russia). Preparations from *T. reesei* and *A. foetidus*

were obtained from the Privolzhski Fermentation Plant (Russia).

Overall cellulolytic activity, measured as filter paper activity (FPA), and activities of different components of cellulase systems (endoglucanase and cellobiase) are summarized in Table 1. The protein content in preparation from *Penicillium* sp. determined by Lowry method (Lowry *et al.*, 1951) was 49%.

Cellulose-containing materials

Short fiber waste material from the paper industry (technical name — skop) and waste after industrial furfural production (cellolignin) were used as the substrates for enzymatic hydrolysis. The total content of polysaccharides in these substrates was determined by quantitative saccharification with 72% sulfuric acid (Saeman *et al.*, 1985). Hemicellulose and cellulose content was established by sequential acid hydrolysis with 2% hydrochloric acid and 80% sulfuric acid (Sinitsyn *et al.*, 1993). The data are summarized in Table 2.

Determination of sugars concentration

Glucose was determined by the glucose oxidase–peroxidase method (Klyosov *et al.*, 1980), using horseradish peroxidase, RZ=0·6 (Reanal, Hungary) and glucose oxidase with specific activity of 380000 U/g (Vilnius Fermentation Plant, Lithuania).

Reducing sugars (RS) were determined by the modified Somogyi–Nelson method (Klyosov *et al.*, 1980).

Mono-, di- and oligosaccharides in hydrolysates were analyzed on a Knauer analytical HPLC instrument with refractive index monitor using Zorbax-NH_2 column (4·6 mm × 25 cm) and acetonitrile–water (70:30) mobile phase.

Table 1. Activities of cellulase preparations

Cellulase preparation	Activity, U/g of preparation		
	FPA	Endoglucanase	Cellobiase
Penicillium sp.	170	760	56
T. reesei	120	820	0·80
A. foetidus	9·0	33	170

Table 2. Content of polysaccharides in skop and cellolignin

Polysaccharides	Content of polysaccharides, %	
	In skop	In cellolignin
Hemicellulose	3	9
Cellulose	83	52
Total polysaccharides	86	61

Determination of enzymatic activities

Filter paper activity was measured by the method described by Ghose (1987) using Whatman No. 1 filter paper (Whatman, UK) as the substrate. Endoglucanase activity was measured viscometrically using medium viscosity carboxymethylcellulose (Sigma, USA) as the substrate (Klyosov *et al.*, 1980). Cellobiase activity was determined by measuring glucose formation from 2 mM cellobiose (Sigma, USA) (Klyosov *et al.*, 1980). All activities were presented in international units (micromole of glycosidic bonds hydrolyzed in 1 min) and calculated per gram of enzymatic preparation (Table 1).

Enzymatic hydrolysis of CCM

Batch hydrolysis of CCM was carried out in 20 ml glass flasks on a shaker at constant temperature. Preweighed substrate was placed in a flask and enzyme solution was added to a final volume of 15 ml. The concentration of substrate, calculated as amount of dry substance per liter of reaction mixture, was varied from 50 to 180 g/l. Enzyme concentration was 2·4–20 g/l or 0·4–3·4 FPU/ml (FPU–filter paper units). In pH optimization experiments 0·1 M Na-acetate buffers (pH 3·5–5·5) or 0·1 M Na-phosphate buffers (pH 6·0) were used. Temperature was varied from 40 to 60°C and intensity of stirring from 0 to 220 rotations per minute (rpm).

To carry out the enzymatic fed-batch hydrolysis of CCM, the substrates (skop and cellolignin) were added to the reaction mixture after each hydrolysis run (12–24 h) to restore their initial concentration (100 and 150 g/l, respectively). Fresh portions of CCM were added 2–3 times until the reaction mixture density prevented agitation. The following enzyme preparations were used in fed-batch experiments: *Penicillium* cellulase (2·0 FPU/ml, cellobiase activity 0·66 U/ml), *T. reesei* cellulase (2·0 FPU/ml, cellobiase activity 0·01 U/ml), combined preparation of *T. reesei* and *A. foetidus* (2·0 FPU/ml, cellobiase activity 0·66 U/ml). The hydrolysis was carried out at pH 4·5, temperature 50°C, stirring intensity 220 rpm.

To monitor glucose, cellobiose and RS formation during hydrolysis, 0·5 ml aliquots of the reaction mixture were taken at different times and analyzed after centrifugation. The yield of sugars was calculated as the percentage of their concentration from the initial substrate concentration. In fed-batch experiments the yield of glucose was determined for each hydrolysis run as the percentage of glucose formed during this run (g/l) from the substrate added during the run.

Determination of CCM reactivity

The initial rate of RS formation under hydrolysis of skop (100 g/l) or cellolignin (150 g/l) by *Penicillium* cellulase (2·0 FPU/ml) at pH 4·5, 50°C and stirring intensity 220 rpm was used as a criteria of substrate reactivity (susceptibility to enzymatic hydrolysis). To monitor skop and cellolignin reactivity during

hydrolysis by *Penicillium* cellulase, the reaction was stopped at 1, 3, 6, 24 or 48 h. The reaction mixture was boiled for 10 min to inactivate the enzymes. Insoluble substrate residue was centrifuged, washed on a glass filter with 0·1 M phosphate buffer (pH 7·0) and then with distilled water. The washed residues of skop and cellolignin were hydrolyzed by fresh cellulase preparations to determine their reactivity. The data were presented as percentage of reactivity of native substrate.

Inhibition of *Penicillium* cellulase by glucose and cellobiose

To study inhibition of *Penicillium* cellulase by reaction products — glucose or cellobiose — these sugars were added to the reaction mixture at the beginning of MCC hydrolysis. The concentration of glucose was varied from 0 to 120 g/l and cellobiose from 0 to 20 g/l.

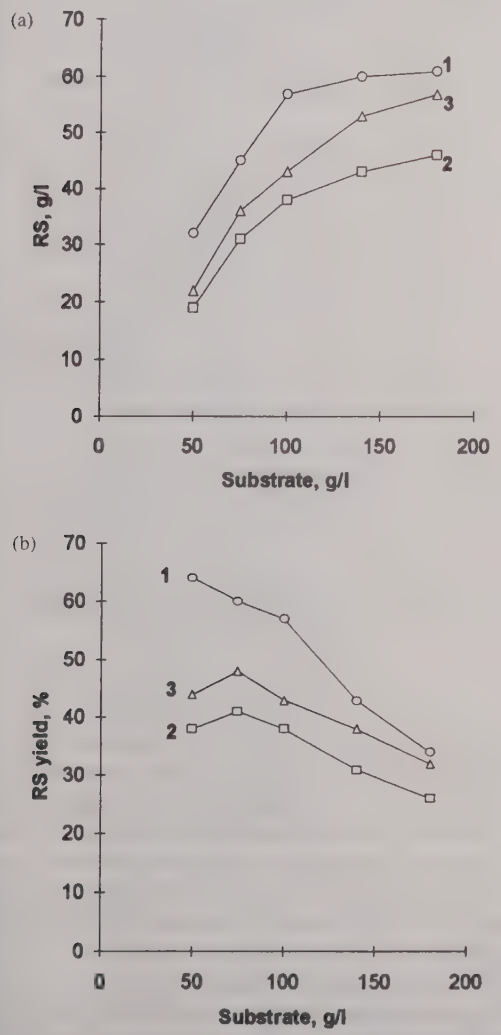

Fig. 1. The influence of substrate concentration on RS concentration (a) and yield of RS (b) in 30 h for skop (1), cellolignin (2), and washed cellolignin (3). Hydrolysis conditions: 1·7 FPU/ml, pH 4·5, 50°C, stirring 220 rpm.

Inactivation of *Penicillium* cellulase

To study inactivation of *Penicillium* cellulase, enzyme solution (1·7 FPU/ml) was incubated at pH 4·5, temperature 50°C, with (220 rpm) or without stirring for 24 h. Aliquots of 1·0 ml were taken at different times to determine FPA and cellobiase activity.

RESULTS AND DISCUSSION

Optimization of enzymatic hydrolysis conditions

The following conditions must be optimized to maximize the efficiency of enzymatic hydrolysis of CCM: substrate and enzyme concentrations, pH, temperature and intensity of stirring. Figures 1–4 and Table

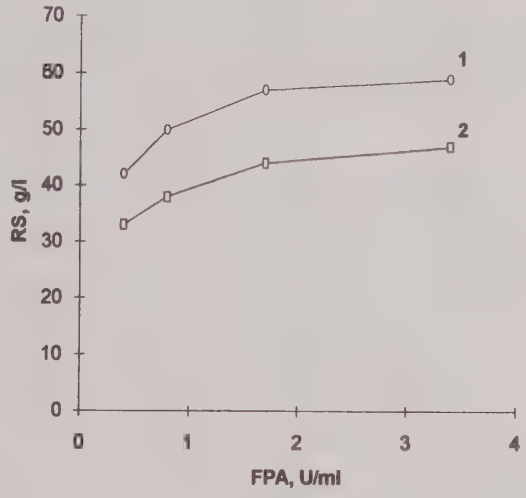

Fig. 2. The influence of *Penicillium* cellulase FPA on RS concentration in 30 h for skop (1) and cellolignin (2). Hydrolysis conditions: skop concentration 100 g/l, cellolignin concentration 150 g/l, pH 4·5, 50°C, stirring 220 rpm.

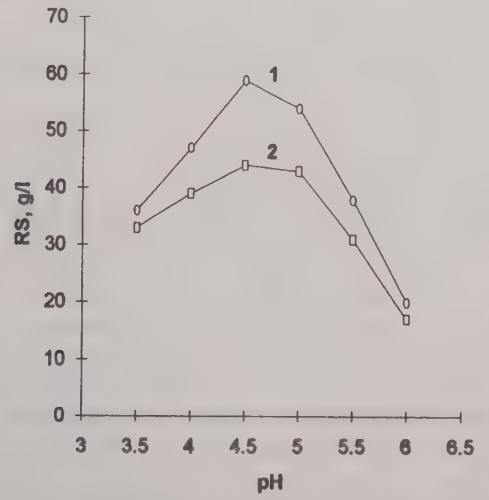

Fig. 3. The influence of pH on RS concentration in 30 h for skop (1) and cellolignin (2). Hydrolysis conditions: 1·7 FPU/ml, skop concentration 100 g/l, cellolignin concentration 150 g/l, 50°C, stirring 220 rpm.

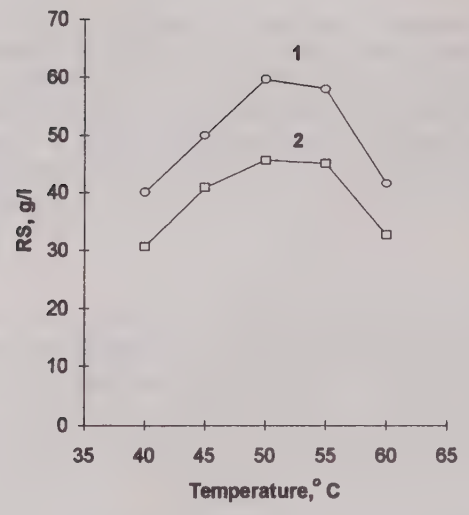

Fig. 4. The influence of temperature on RS concentration in 30 h for skop (1) and cellolignin (2). 1·7 FPU/ml, skop concentration 100 g/l, cellolignin concentration 150 g/l, pH 4·5, 50°C, stirring 220 rpm.

Table 3. Hydrolysis of skop and cellolignin by *Penicillium* cellulase at different stirring intensity

Stirring intensity, rpm	Skop		Cellolignin	
	Initial rate of glucose formation, g/l.h	Glucose concentration after 24 h, h/l	Initial rate of glucose formation, g/l.h	Glucose concentration after 24 h, g/l
0	6·6	47	0·3	8
20	6·6	49	0·6	13
45	8·4	50	0·9	18
220	12·6	51	12·3	43

Hydrolysis conditions: 2·0 FPU/ml, skop concentration 100 g/l, cellolignin concentration 150 g/l, pH 4·5, 50°C.

3 present the results of skop and cellolignin hydrolysis by *Penicillium* cellulase under different conditions.

The results of Fig. 1(a) show that the concentration of RS after 30 h hydrolysis of skop almost doubled as substrate concentration increased from 50 to 100 g/l. Further increasing skop concentration up to 140 and 180 g/l did not significantly affect the concentration of sugars but drastically reduced the yield of RS from 57% at 100 g/l to 34% at 180 g/l [Fig. 1(b)]. Also, at skop concentrations higher than 100 g/l it was difficult to agitate the reaction mixture and to separate glucose solution from the insoluble residue of substrate at the end of hydrolysis. We conclude that the optimal concentration of skop was 100 g/l.

The RS concentration after 30 h of cellolignin hydrolysis increased when substrate concentration increased up to 180 g/l [Fig. 1(a)]. However, when cellolignin concentration was higher than 140–150 g/l, the reaction mixture was difficult to agitate. For this reason, the optimal concentration of cellolignin was established as 150 g/l. It should be noted that the yield of RS and, therefore, the degree of cellolignin conversion slightly decreased at substrate concentrations higher than 75 g/l [Fig. 1(b)]. However, that was not taken into account, because cellolignin is a low-cost waste material of furfural production. The high temperature and pressure conditions during production of furfural from wood can result in formation of sugars and lignin destruction products in cellolignin. These products can inhibit cellulolytic enzymes and could be eliminated by washing of cellolignin with water (Sinitsyn *et al.*, 1982). Figure 1(a) shows that the RS concentration after 30 h of hydrolysis for unwashed cellolignin was 81–88% compared to washed substrate. Since the difference in RS concentration was not significant, unwashed cellolignin was used for further experiments.

As shown in Fig. 2 the optimal level of FPA of *Penicillium* cellulase was 1·7 U/ml for hydrolysis of both skop (100 g/l) and cellolignin (150 g/l). Further increase of FPA in reaction mixture (up to 3·4 U/ml) did not significantly influence the concentration of sugars.

The pH and temperature optima based on the RS concentration after 30 h of hydrolysis of skop and cellolignin were pH 4·5 (Fig. 3) and 50°C (Fig. 4).

We investigated the effect of agitation intensity on the reaction mixture on the efficiency of skop and cellolignin hydrolysis (Table 3). The agitation factor was found to influence the efficiency of enzymatic hydrolysis of these two substrates differently. For cellolignin, the increase in agitation intensity from 0 to 220 rpm intensified hydrolysis: the initial rate of glucose formation increased up to 41-fold and glucose concentration after 24 h of hydrolysis up to 5-fold. On the contrary, for skop hydrolysis the same increase in agitation intensity led to only minor changes in the parameters of the process: the initial rate of glucose formation increased only 1·9-fold and glucose concentration after 24 h of hydrolysis remained practically the same (Table 3).

A reason for different effects of agitation intensity on the efficiency of skop and cellolignin hydrolysis was probably differences in their structure. Particles of cellolignin were dense and tend to sedimentation in solution if stirring was insufficient. Intensification of agitation prevented sedimentation and increased the accessibility of the substrate surface to enzymes. In contrast, skop was fibrous, light material, its distribution in the reaction mixture was uniform and did not depend on intensity of stirring.

Varying skop and cellolignin concentrations in the reaction mixture permitted determination of the kinetic parameters of enzymatic hydrolysis of these substrates — K_m and V_{max} (Table 4). The results (initial rate of RS formation as a function of initial substrate concentration) were linearized in Line-

weaver–Burk plots. The Michaelis constants were calculated with and without consideration of cellulosic fraction content in the substrates (K'_m and K_m) and were found to be 49 and 59 g/l for skop and 65 and 125 g/l for cellolignin. The V_{max} was normalized to 1 g of cellulase preparation (V_{max}) as well as to 1 g of protein (V'_{max}). The values of V_{max} and V'_{max} of RS formation were 170 and 347 μM/min.g for cellolignin (Table 4).

Enzymatic hydrolysis of skop and cellolignin under periodic feeding of substrates (fed-batch process)

Since the cost of glucose enzymatically-produced from CCM is significantly determined by the cost of the enzyme preparation (Mandels, 1985), it is necessary to optimize the mode of hydrolysis to the most complete utilization of enzyme. One approach is to hydrolyze several portions of substrate with one portion of enzyme preparation.

Two basic methods can realize this approach. The first one supposes that for more complex utilization of cellulases, the enzyme recovery step should come at the end of each hydrolysis run. Recovered enzyme can then be used for hydrolysis of a new portion of substrate (Clesceri *et al.*, 1985; Singh *et al.*, 1991). The second method suggests periodic feeding of substrate during the fed-batch hydrolysis process (Ramos *et al.*, 1993). It was shown (Mandels *et al.*, 1971; Ramos *et al.*, 1993) that the second method is more efficient and easier to use. Therefore we decided to perform feasibility studies for a fed-batch saccharification process of skop and cellolignin by different cellulases (see Methods). Three preparations (*Penicillium* sp., *T. reesei* and a combined preparation of *T. reesei* and *A. foetidus*) with 2·0 FPU/ml were used for these experiments.

The best results of fed-batch hydrolysis were obtained with *Penicillium* cellulase (Table 5, #1). The intial rate of glucose formation from skop and cellolignin during hydrolysis by *Penicillium* cellulase

Table 4. Kinetic parameters of CCM hydrolysis by *Penicillium* cellulase

Substrate	Initial substrate concentration, g/l	Initial rate of RS formation, g/l.h	K_m, g/l	K'_m, g/l	V_{max}, μM/min per g of preparation	V'_{max}, μM/min per g of protein
Skop	50	820				
	75	940				
	100	1140	59	49	170	347
	140	1280				
	180	1280				
Cellolignin	50	600				
	75	750				
	100	890	125	65	212	433
	140	1160				
	180	1220				

Hydrolysis conditions: 1·7 FPU/ml, pH 4·5, 50°C, stirring intensity 220 rpm.
Values of K'_m were determined based on content of cellulose in skop (83%) and cellolignin (52%).
Value of V'_{max} were determined based on 49% protein content in cellulase preparation.

Table 5. Hydrolysis of skop and cellolignin by cellulase preparations under periodic feeding of substrates

Cellulase preparation	Period of hydrolysis								
	#1, 24 h			#2, 24 h			#3, 24 h		
	Initial rate of glucose formation, g/l.h	Glucose, g/l	Glucose yield, %	Initial rate of glucose formation, g/l.h	Glucose, g/l	Glucose yield, %	Initial rate of glucose formation, g/l.h	Glucose, g/l	Glucose yield, %
Hydrolysis of skop									
Penicillium cellulase	12·0	50	50	3·0	88	38	2·4	110	22
T. reesei	2·4	37	37	0·6	59	22	—	—	—
T. reesei+*A. foetidus*	7·8	45	45	1·8	70	25	—	—	—
Hydrolysis of cellolignin									
Penicillium cellulase	9·6	49	33	3·0	85	24	1·8	102	11
T. reesei	1·8	23	15	0·6	34	7·3	—	—	—
T. reesei+*A. foetidus*	6·0	40	27	1·8	56	11	—	—	—

See hydrolysis conditions in Methods.

in each 24 h run was approximately 5 times higher than for *T. reesei* (Table 5, #2) and 1·5 times higher than for combined preparations *T. reesei* and *A. foetidus* (Table 5, #3). Use of *Penicillium* preparation resulted in a higher yield of glucose in each run and allowed more concentrated glucose solutions. It was possible to carry out three hydrolysis runs with *Penicillium* cellulase instead of two runs for other preparations. As a result, 102–110 g/l glucose solutions were obtained during 72 h fed-batch hydrolysis of skop and cellolignin by *Penicillium* cellulase.

Low efficiency of skop and cellolignin hydrolysis by cellulase from *T. reesei* can be explained by insufficient cellobiase activity in this preparation (Table 1). When *T. reesei* preparation was combined with *A. foetidus* preparation to obtain the same level of cellobiase activity as in *Penicillium* cellulase (0·66 U/ml), the efficiency of hydrolysis increased (Table 5), but still remained lower compared to *Penicillium* preparations. This fact can be explained by the higher operational stability of *Penicillium* cellulase or its lower inhibition by hydrolysis products (glucose or cellobiose).

The objective of further optimization of CCM fed-batch hydrolysis was to reduce the overall hydrolysis time. The kinetics of glucose formation from skop and cellolignin show that 75–80% of the maximum level of glucose concentration in each run is achieved in the first 10–12 h of hydrolysis. Hence we decided to add new portions of substrates after this time. In this case one portion of *Penicillium* preparation (2·0 FPU/ml) was able to hydrolyze three portions of skop or cellolignin in 48 h and allowed to obtain 93–98 g/l glucose solution (Table 6 and Fig. 5). Final cellobiose concentration was about 19 g/l (16% from total concentration of glucose and cellobiose in hydrolysate) for skop and 12 g/l (11% from total concentration of these sugars) for cellolignin (Fig. 5).

The use of fed-batch techniques under these conditions allowed us to reduce enzyme consumption compared to batch process: enzyme loading per gram of substrate was decreased 1·7 times for skop and 1·4 times for cellolignin (Table 7). Productivity based on final glucose concentration was practically the same for both techniques of hydrolysis (about 2·1 g/l h for skop and 1·9 g/l h for cellolignin), but final glucose concentration for fed-batch hydrolysis was almost twice as high as for batch hydrolysis (Table 7). Fed-batch technique also provided a higher glucose yield compared to batch technique (Table 7).

During fed-batch hydrolysis of skop and cellolignin each new portion of substrate was hydrolyzed

Table 6. Hydrolysis of skop and cellolignin by *Penicillium* preparation under periodic feeding of substrates

Substrate	Period of hydrolysis								
	#1, 12 h			#2, 12 h			#3, 24 h		
	Initital rate of glucose formation, g/l.h	Glucose, g/l	Glucose yield, %	Initial rate of glucose formation, g/l.h	Glucose, g/l	Glucose yield, %	Initial rate of glucose formation, g/l.h	Glucose, g/l	Glucose yield, %
Skop	12·0	43	43	7·8	73	30	6·0	98	31
Cellolignin	9·6	38	25	3·6	64	17	3·0	93	13

See hydrolysis conditions in Methods.

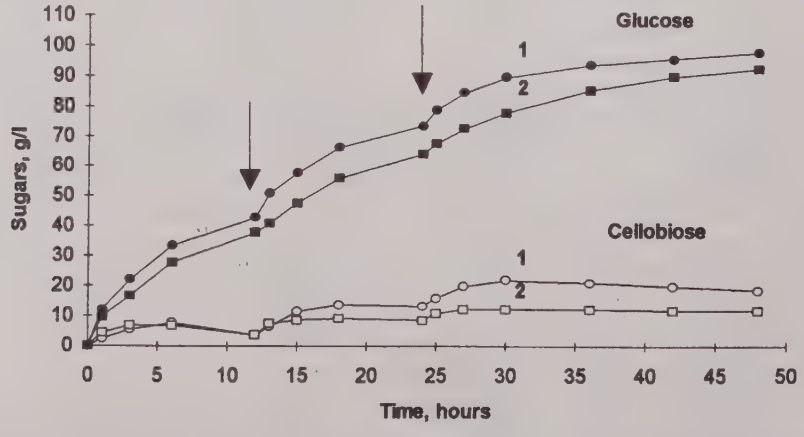

Fig. 5. Hydrolysis of skop (1) and cellolignin (2) by *Penicillium* cellulase under periodic feeding of substrates (addition of each new portion of substrate is marked by arrow). See hydrolysis conditions in Methods.

Table 7. Comparison of batch and fed-batch hydrolysis of skop and cellolignin by *Penicillium* preparation

Parameter	Skop		Cellolignin	
	Batch hydrolysis, 24 h	Fed-batch hydrolysis, 48 h	Batch hydrolysis, 24 h	Fed-batch hydrolysis, 48 h
Substrate concentration, g/l	100	173[a]	150	213[a]
Enzyme loading, FPU/g substrate	20	11·6	13·3	94
Glucose concentration, g/l	51	98	43	93
Glucose yield, %	51	57[b]	29	44[b]
Degree of cellulose conversion to glucose, %[c]	61	68	55	84

See hydrolysis conditions in Methods.
[a]Total concentration of substrate added to the reaction mixture during fed-batch hydrolysis.
[b]Percentage of final glucose concentration in fed-batch hydrolysis from total substrate concentration.
[c]Calculated as percentage of final glucose concentration from cellulose concentration in reaction mixture (see cellulose content in skop and cellolignin, Table 2).

with less efficiency than the previous one. For instance, for skop hydrolysis by *Penicillium* preparation, the initial rate of glucose formation in the last (third) run was half that in the first run and the yield of glucose was 1·4 times lower (Table 6). For cellolignin, the corresponding parameters decreased 3·2 and 1·9 times from the first to the last run (Table 6). The following reasons could explain this phenomenon: decrease of susceptibility of substrates to enzymatic attack; inhibition of enzymes by glucose and cellobiose (their concentration in the reaction mixture was rather high), and inactivation of enzymes during 48–72 h hydrolysis.

The role of various factors in decrease of the efficiency of CCM hydrolysis

The rate of hydrolysis of skop and cellolignin by *Penicillium* cellulase is a function of degree of substrates conversion — the higher degree of conversion the slower the rate of reaction. Three main reasons should be considered to explain this phenomenon: decrease of reactivity of substrates, inhibition of enzymes by reaction products (glucose and cellobiose) and inactivation of enzymes. The yield of glucose could be also reduced due to reactions of transglycosylation, which lead to formation of oligosaccharides. We evaluated the role of all these factors in the hydrolysis of both substrates by *Penicillium* cellulase.

It was shown that decreased skop and cellolignin reactivity during hydrolysis significantly affected the efficiency of their saccharification by *Penicillium* cellulase. Corresponding data are presented in Fig. 6. The reactivity of both substrates rapidly decreased to 67% for skop and to 38% for cellolignin during the first 6 h of hydrolysis [Fig. 6(a)]. This can be explained by fast initial consumption of readily hydrolyzable fractions of these substrates. The decreased susceptibility to enzymatic hydrolysis was different for skop and cellolignin. For instance, at 40% yield of RS [Fig. 6(b)], the reactivity of skop decreased 1·3 times and the reactivity of cellolignin 4·5 times. This may be explained by the screening effect of lignin molecules, which limit the accessibility of the cellulose surface to cellulolytic enzymes (Klyosov *et al.*, 1985).

The product inhibition decreased the efficiency of skop and cellolignin hydrolysis. The initial rate of glucose formation significantly decreased when glucose [Fig. 7(a)] or cellobiose [Fig. 7(b)] were added to the reaction mixture at the beginning of hydrolysis. For example, at a glucose concentration of 5 g/l the initial rate of hydrolysis of substrates decreased 52–61%, at a concentration of 80 g/l, to 7–9% [Fig. 7(a)]. At a cellobiose concentration of 5 g/l the initial rate of hydrolysis of skop and cellolignin decreased to 37 and 57%, respectively, to 25 and 35% at a concentration of 20 g/l.

The stability of *Penicillium* cellulase was rather high: when the enzyme solution was incubated for 24 h without stirring at pH 4·5 and 50°C the loss of FPA and cellobiose activity was 5 and 12%, respectively. Under stirring (220 rpm) the loss of these activities was 8 and 16%. Therefore, the contribution of inactivation of *Penicillium* enzymes to decrease of enzymatic hydrolysis efficiency was not significant.

Transglycosylation catalyzed by cellobiose (Gusakov *et al.*, 1984) can decrease the glucose yield during cellulose saccharification because of formation of different oligosaccharides from glucose and cellobiose. Indeed, chromatographic analysis of reaction mixture after 24 h hydrolysis of skop (100 g/l) by *Penicillium* cellulase (2·0 FPU/ml) has shown that it contains the products of transglycosylation: gentiobiose and isocellotriose. However, their concentrations in hydrolysates were only 1·5 and 0·5 g/l, respectively (whereas the concentration of glucose was 50 g/l). Similar results were obtained for hydrolysis of cellolignin. Therefore, transglycosylation did not significantly reduce the yield of glucose during enzymatic hydrolysis of CCM.

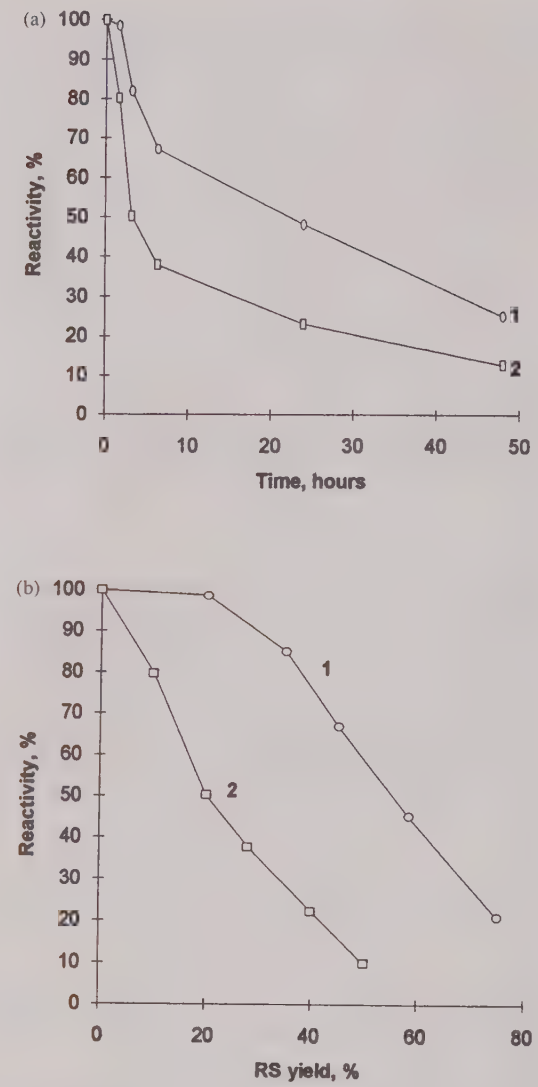

Fig. 6. Decrease of skop(1) and cellolignin (2) reactivity during hydrolysis by *Penicillium* cellulase vs hydrolysis time (a) or RS yield (b). Hydrolysis conditions: 2·0 FPU/ml, skop concentration 100 g/l, cellolignin concentration 150 g/l, pH 4·5, 50°C, stirring 220 rpm.

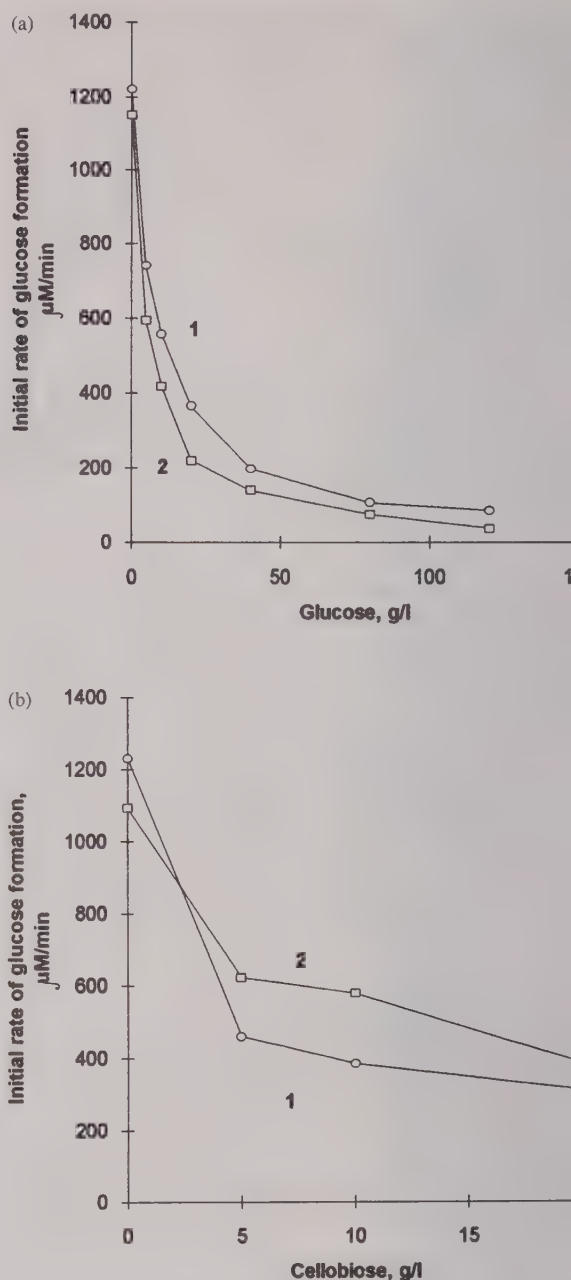

Fig. 7. Decrease of the initial rate of glucose formati during hydrolysis of skop (1) and cellolignin (2) by *P icillium* cellulase in presence of glucose (a) and cellobi (b). Hydrolysis conditions as in Fig. 6.

CONCLUSIONS

The conditions of skop and cellolignin hydrolysis by *Penicillium* cellulase in a batch reactor have been evaluated. The optimal conditions have been found: 1·7 FPU/ml, concentration of skop 100 g/l, cellolignin 150 g/l, pH 4·5, temperature 50°C. Agitation had different influences on the efficiency of hydrolysis of these two substrates. For cellolignin, increased agitation intensity enhanced hydrolysis, whereas for skop the role of this factor was insignificant.

The feasibility studies for fed-batch saccharification of skop and cellolignin by three cellulases (*Penicillium* sp., *T. reesei* and a combined preparation of *T. reesei* and *A. foetidus*) have been performed. The best result of fed-batch hydrolysis was obtained with *Penicillium* cellulase. One portion of this preparation (2·0 FPU/ml, cellobiase activ 0·66 U/ml) was able to hydrolyze three portions skop or cellolignin and to produce 98 and 93 glucose solutions in 48 h, respectively. At the sa time one portion of the combined preparation (w the same FPA and cellobiase activity) carried only two hydrolysis runs and produced 70 and 56 glucose solutions in 48 h.

The following advantages of fed-batch hydroly compared to batch hydrolysis have been demo strated: lower enzyme loading per gram of substr (only 11·6 FPU/gl for skop and 9·4 FPU/gl for cel

lignin) and higher final glucose concentration at the same productivity of both processes (about 2 g/l h).

The role of different factors in decreased efficiency of CCM hydrolysis by *Penicillium* cellulase has been evaluated. Inactivation of enzymes and transglycosylation did not play an important role in this process, but decreased substrate susceptibility to enzymatic hydrolysis in the course of the reaction; inhibition of enzymes by glucose and cellobiose can also be considered as the main factors leading to a decrease of the hydrolysis efficiency.

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COMPARATIVE EVALUATION OF HYDROLYTIC EFFICIENCY TOWARD MICROCRYSTALLINE CELLULOSE OF *PENICILLIUM* AND *TRICHODERMA* CELLULASES

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Abstract

Composition, stability at different pH and temperatures and hydrolytic efficiency toward microcrystalline cellulose (MCC) of Penicillium *cellulase were evaluated. The pH optimum (4·5–5·0) of filter paper activity (FPA) and temperature optimum (50–55°C) were determined. Maximum stability of FPA was observed at pH 4·0–5·0 and temperature 40–50°C. A minor contribution of stirring (3–4%) in inactivation of* Penicillium *preparation was shown.*

Kinetic parameters of MCC hydrolysis by Penicillium *cellulase were determined at optimal conditions (pH 4·5, 50°C):* K_m*=50 g/l,* V_{max}*=330 μM/min per gram of protein.*

More effective saccharification of MCC by Penicillium *preparations compared to* Trichoderma reesei *cellulases with the same level of total cellulolytic activity (FPA=1·0 U/ml) was demonstrated. Glucose concentration in 24 h hydrolysate was 41 g/l for* Penicillium *cellulase, 15 g/l for* T. reesei *(Privolzhski Fermentation Plant) and 26 g/l for* T. reesei *(Genencor). Hydrolysates of* Penicillum *cellulase contained only 9% of cellobiose compared to 24–40% of cellobiose for* T. reesei *cellulases. Factors contributing to superior properties of* Penicillium *cellulase — higher level of cellobiase activity and higher operational stability — are discussed.*

Key words: Cellulase, *Penicillium* sp., *Trichoderma reesei*, enzymatic hydrolysis, microcrystalline cellulose.

INTRODUCTION

Cellulose is the world's most abundant renewable carbon source that can be hydrolyzed to glucose by microbial enzymes. The most frequently reported source of cellulolytic enzymes is the fungus *Trichoderma reesei* (Persson *et al.*, 1991). However, cellulases from this source cannot be considered as 'technological' ones for hydrolysis of cellulose because of some drawbacks — relatively low specific activity, low thermostability and high sensitivity to product inhibition (Mandels, 1985; Klyosov, 1988). Besides, low cellobiase activity of enzyme systems from *Trichoderma reesei* leads to incomplete hydrolysis of cellobiose to glucose, accumulation of cellobiose in reaction mixture and, as a result, to strong inhibition of the enzymes (Mandels, 1985; Holtzapple *et al.*, 1990).

There has been an intensive search for new microorganisms which produce cellulolytic enzymes of higher specific activities and greater efficiency. The objective of this study is to compare cellulase preparations from a new mutant strain of *Penicillium* sp. with two commercial preparations from *T. reesei* and to evaluate the influence of cellobiase activity and operational stability of these cellulases on the efficiency of microcrystalline cellulose hydrolysis.

METHODS

Cellulases

An enzyme preparation from *Penicillium* sp. (freeze-dried culture filtrate) was provided by the Institute of Biochemistry and Physiology of Microorganisms (Russia). Preparations from *T. reesei* and *Aspergillus foetidus* were obtained from the Privolzhski Fermentation Plant — PFP (Russia). Cytolase 300 (dry enzyme preparation from *T. reesei*) was obtained from Genencor (CA, USA) and Novozym 180 (culture filtrate of *Aspergillus niger*) from Novo (CT, USA).

The overall cellulolytic activity, measured as filter paper activity (FPA) and activities of different components of these cellulase systems (endoglucanase and cellobiase), is summarized in Table 1.

Substrates and reagents

Microcrystalline cellulose (MCC, Chemapol, Czech Republic) was used as a model substrate to study the kinetics of enzymatic hydrolysis with cellulases. The following substrates from different suppliers were used to determine enzymatic activities: Whatman No. 1 filter paper (Whatman, UK); sodium salt of carboxymethylcellulose (CMC, medium viscosity),

cellobiose, p-nitrophenyl-β-D-glucoside (p-NPh-β-D-glucoside), p-NPh-β-D-cellobioside, p-NPh-β-D-lactoside, β-glucan from barley and xylan from birchwood (Sigma, USA); Avicel-cellulose (type PH) and amylose (MM 150000) (Serva, Germany). Amorphous cellulose was obtained by treatment of microcrystalline cellulose with phosphoric acid (Sinitsyn *et al.*, 1991).

D-Glucose (Sigma, USA) was used as a standard in HPLC and δ-gluconolacton (Sigma, USA) as an inhibitor of cellobiase.

Glucose oxidase with specific activity of 380000 U/g (Vilnius Fermentation Plant, Lithuania) and horseradish peroxidase, RZ=0·6 (Reanal, Hungary) were used for glucose analysis.

Determination of protein and sugars concentration

The protein content in the cellulase preparations was determined by Lowry method (Lowry *et al.*, 1951). Data are summarized in Table 1.

Glucose concentration was measured by the glucose oxidase–peroxidase method and reducing sugars (RS) by the modified method of Somogyi–Nelson (Klyosov *et al.*, 1980) and the dinitrosalicylic acid method (Ghose, 1987).

Mono-, di- and oligosaccharides in hydrolysates were analyzed on a Knauer analytical HPLC instrument with a refractive index monitor using Zorbax–NH_2 column (4·6 mm × 25 cm) and acetonitrile–water (70:30) mobile phase.

Determination of enzymatic activities

Filter paper activity was measured according to the method published by Ghose (1987). CMCase activity, avicelase activity and activity toward amorphous cellulose were estimated as the initial rate of RS formation during hydrolysis of CMC (10 g/l), Avicel-cellulose (5 g/l) or amorphous cellulose (5 g/l), respectively. Exoglucosidase activity (the total activity of glucose-producing enzymes excluding cellobiase) was determined as the initial rate of glucose formation during hydrolysis of CMC (10 g/l) in the presence of δ-gluconolacton (20 g/l); cellobiase activity was determined as the initial rate of glucose formation during hydrolysis of 2 mM cellobiose. The total activity of cellobiose-producing enzymes (cellobiohydrolase and/or endoglucanase) was determined as the initial rate of cellobiose formation during hydrolysis of filter paper (33 g/l) in the presence of δ-gluconolation (20 g/l). Endoglucanase activity was measured viscometrically with 5 g/l CMC (Sinitsyn *et al.*, 1990).

Activities toward p-NPh-β-D-glucoside (β-glucosidase), p-NPh-β-D-cellobioside and p-NPh-β-D-lactoside were determined as the initial rate of p-nitrophenol formation during hydrolysis of these substrates (0·5 mM). Reactions were stopped at 10 min by addition of 0·5 ml of 1 M Na_2CO_3 to 1 ml of the reaction mixture. Optical density readings were taken at 400 nm.

β-Glucanase, xylanase, and amylase activities were measured as the initial rate of RS formation during hydrolysis of β-glucan (5 g/l), xylane (5 g/l), and amylose (10 g/l), respectively.

Enzymatic activities were determined at pH 4·5 and 40°C — except amylase activity (pH 6·0), FPA (50°C) and total activity of cellobiose-producing enzymes (50°C).

All activities were expressed in international units (micromole of glycoside bonds of substrate hydrolyzed in 1 min) and normalized to 1 g of preparation of 1 g of protein (Table 2).

Table 1. Specific activities of cellulase preparations

Cellulase preparations	Protein, %	Specific activity, U/g of protein		
		FPA	Endoglucanase	Cellobiase
Penicillium sp.	49	350	1550	110
T. reesei (PFP)	38	320	2160	2·1
A. foetidus (PFP)	6·5	140	510	2550
T. reesei (Genencor)	24	410	4790	37
Concentrated culture filtrate *A. niger* (Novo)	65[a]	15·5	68	9350

[a] Concentration of protein, g/l.

Enzymatic hydrolysis of MCC

Enzymatic hydrolysis of MCC was carried out in 20 ml glass flasks on a shaker (stirring intensity 220 rotations per min, rpm) at pH 4·5 (0·1 M Na-acetate buffer) and 50°C. Preweighed substrate was placed in a flask and enzyme solution was added to a final volume of 15 ml. The substrate concentration was 100 g/l, total cellulase activity was 1·0 FPU/ml (FPU — filter paper units). The composition of combined enzyme preparations *T. reesei* (PFP)+*A. foetidus* (PFP) or *T. reesei* (Genencor)+*A. niger* (Novo) was chosen to provide a ratio of FPA to cellobiase activity equal to 3·0, as in *Pencillium* preparations. It was achieved by mixing *Trichoderma* and *Aspergillus* preparations from PFP in 3·25 to 1 w/w proportion or by adding of 0·12 volume of *A. niger* concentrated culture filtrate to 250 volumes of 8·7 g/l solution of *T. reesei* (Genencor). To monitor glucose and cellobiose formation during hydrolysis, 0·5 ml aliquots of the reaction mixture were taken at different times and analyzed after centrifugation. To determine the kinetic parameters (K_m and V_{max}) of MCC hydrolysis by *Penicillium* cellulase, substrate concentration was varied from 20 to 100 g/l, enzyme concentration was 2·0 FPU/ml.

RESULTS AND DISCUSSION

Activities of *Penicillium* cellulase system

The overall cellulolytic activity (FPA), as well as activities of different components (endoglucanase

Table 2. Activities of *Penicillium* cellulase preparations

#	Activity	U/g of preparation	U/g of protein
1	FPA	170	350
2	CMC-ase	1050	2140
3	Avicelase	180	370
4	Toward amorphous cellulose	270	550
5	Endoglucanase	760	1550
6	Exoglucosidase	50	100
7	Total activity of cellobiose-producing enzymes	110	220
8	Cellobiase	56	110
9	β-Glucosidase	450	920
10	Toward p-NPh-β-D-cellobioside	100	200
11	Toward p-NPh-β-D-lactoside	43	88
12	β-Glucanase	1170	2390
13	Xylanase	2220	4530
14	Amylase	17	35

and cellobiase) of cellulase preparations from different fungi (*Penicillium*, *Trichoderma, Aspergillus* sp.) are summarized in Table 1.

Specific FPA of *Penicillium* cellulase was about the same as *Trichoderma* cellulases, 2·5 times higher than for preparations from *A. foetidus* (PFP) and 23 times higher than for concentrated culture filtrate of *A. niger* (Novo). Endoglucanase activity of *Penicillium* cellulase was 3·1 times lower than for *T. reesei* (Genencor) and 1·4 times lower than for *T. reesei* (PFP), but three times higher compared to *A. foetidus* (PFP) and 23 times higher compared to *A. niger* (Novo). Cellobiase activity of *Penicillium* cellulase was 52 times higher than for *T. reesei* (PFP) and three times higher than for *T. reesei* (Genencor), but significantly lower than for *Aspergillus* preparations (Table 1).

Detailed information about different kinds of enzymatic activities of *Penicillium* preparation (cellulolytic activities, #1–12, as well as activities of related enzymes, #13–14) are summarized in Table 2. Some of those activities represent the total activity of the cellulase system (#1–4) and others represent activities of individual enzymes (#5, 8, 9). It should be mentioned that *Penicillium* preparations demonstrated relatively high CMC-ase, endoglucanase, β-glucanase and xylanase activities.

Activity and stability of *Penicillium* cellulase at different pH and temperatures

The pH and temperature profiles of *Penicillium* cellulase FPA show common features for fungal cellulases (Klyosov *et al.*, 1980) optimal pH, 4·5–5·0 [Fig. 1(a)], and temperature, 50–55°C [Fig. 1(b)].

Effect of pH and temperature on *Penicillium* cellulase stability during 24 h incubation monitored by FPA changes are shown in Fig. 2. At pH 4·0–5·0 and 50°C FPA did not reduce significantly in 24 h; at pH 6·0 FPA decreased to 78% from its initial level in 6 h and to 65% in 24 h; at pH 7·0 only 19% of initial FPA in 6 h and 9% in 24 h remained in solution

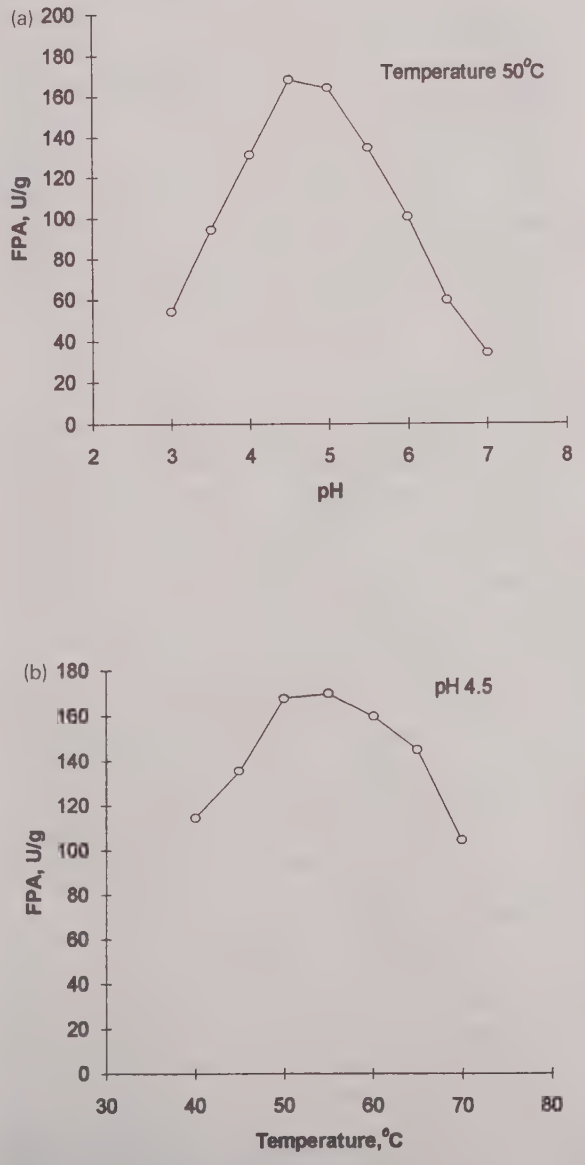

Fig. 1. The pH (a) and temperature (b) profiles of FPA of *Penicillium* preparations.

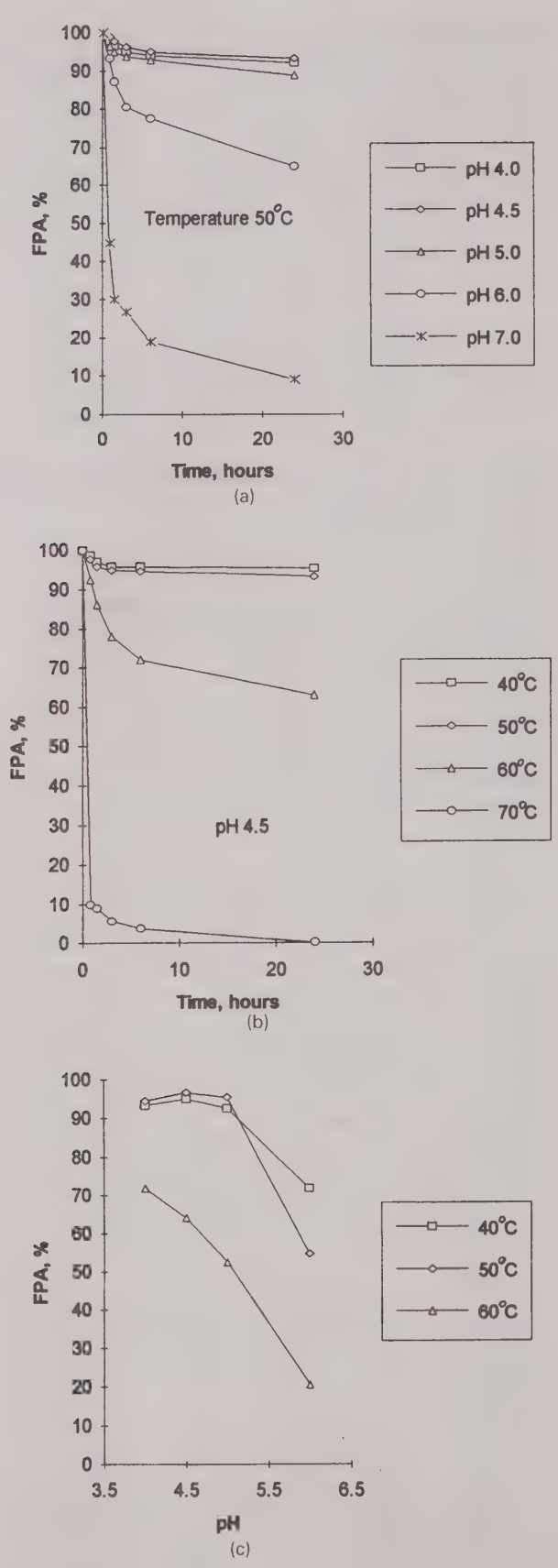

Fig. 2. Effect of pH (a) and temperature (b) on stability of FPA of *Penicillium* preparation during 24 h incubation without stirring. (c) Residual FPA after 24 h incubation at different pH and temperatures.

[Fig. 2(a)]. At pH 4·5 and temperatures of 40 and 50°C there were no significant changes of FPA in 24 h. At higher temperatures drastic inactivation was observed — at 60°C FPA decreased to 72% in 6 h and to 63% in 24 h, at 70°C FPA decreased to 10% in 1 h [Fig. 2(c)] represents residual FPA after incubation of *Penicillium* cellulase during 24 h at different pH and temperatures. Maximum stability of FPA was observed at pH 4·0–5·0 and temperature 40–50°C.

It was shown that stirring (220 rpm) does not affect FPA and cellobiase activity from *Penicillium* preparation significantly. The loss of FPA and cellobiase activity during 24 h incubation at pH 4·5, and 50°C was 5 and 12% without stirring and 8 and 16% with stirring. Therefore the contribution of stirring in inactivation was only 3 and 4%, respectively.

At optimal conditions (pH 4·5, 50°C) we determined the kinetic parameters (K_m and V_{max}) of MCC hydrolysis. The initial rate of glucose formation was measured as a function of initial substrate concentration and the data were linearized in Lineweaver–Burk plots (Fig. 3). The K_m and V_{max} values were calculated and found to be K_m=50 g/l and V_{max}=330 μM/min per gram of protein.

Comparative evaluation of MCC hydrolysis by different cellulase preparations

The following enzyme preparations were used for hydrolysis of MCC: *Penicillium* cellulase (#1), two commercial cellulases from *Trichoderma reesei* (#2, #4) and two combined preparations (#3, #5) consisting of *Trichoderma* and *Aspergillus* cellulases

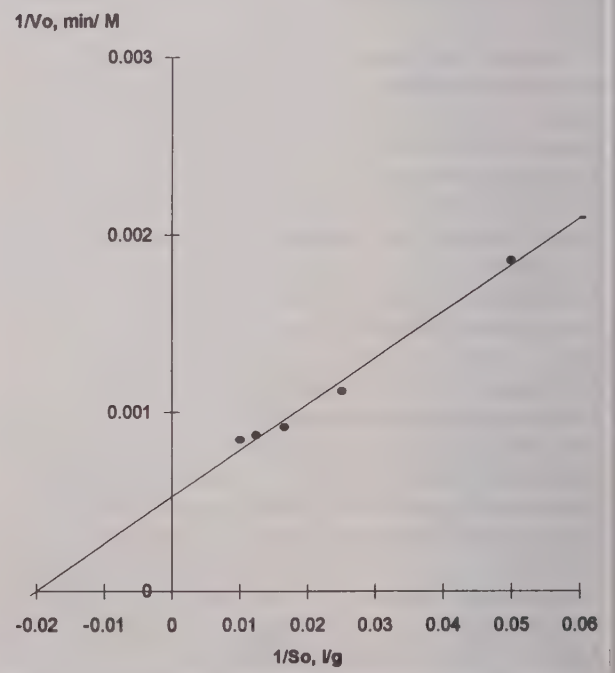

Fig. 3. Determination of kinetic parameters of MCC hydrolysis by *Penicillium* cellulase in Lineweaver–Burk plots. Conditions: 2·0 FPU/ml, pH 4·5, 50°C, 220 rpm.

Table 3. MCC hydrolysis by individual and combined cellulase preparations

#	Cellulose preparations	Enzyme concentration, g/l	Cellobiase activity, U/ml	Initial rate of glucose formation, g/l-h	Concentration of sugars in 24 h hydrolysate, g/l		Composition of 24 h hydrolysate, %	
					Glucose	Cellobiose	Glucose	Cellobiose
1	*Penicillium* sp.*	5·9	0·33	10·4	41	4	91	9
2	*T. reesei* (PFP)	8·2	0·01	1·3	15	10	60	40
3	*T. reesei* (PFP)+ *Asp. foetidus* (PFP)*	9·1	0·33	6·4	32	4	89	11
4	*T. reesei* (Genencor)	10·2	0·09	10·1	26	8	76	24
5	*T. reesei* (Genecor)+ *Asp. niger* (Novo)*	8·7	0·33	8·5	37	4	90	10

See hydrolysis conditions in Methods.
*Ratio between FPA and cellobiase for #1, #3 and #5 preparations was equal 3·0.
See composition of combined enzyme preparations in Methods.

Table 4. Inactivation of cellulase preparations at pH 4·5 and 50°C

#	Cellulase preparations	% FPA, 1 h		% FPA, 6 h		% FPA, 24 h	
		Without stirring	Under stirring[a]	Without stirring	Under stirring[a]	Without stirring	Under stirring[a]
1	*Penicillium* sp.	98	97	96	93	95	92
2	*T. reesei* (PFP)	94	92	81	72	65	30
3	*T. reesei* (PFP)+*A. foetidus* (PFP)	91	80	68	58	60	28
4	*T. reesei* (Genencor)	92	91	89	87	52	42
5	*T. reesei* (Genencor)+*A. niger* (Novo)	92	91	89	87	53	44

[a] Stirring intensity 220 rpm.

(Table 3). The ability of enzymes to hydrolyze MCC was compared using such key parameters as the initial rate of glucose formation and concentration of glucose and cellobiose after 24 h of hydrolysis. For accurate comparison the FPA of all preparations was adjusted to 1·0 U/ml. Cellobiase activity for *Penicillium* cellulase and combined preparations was also adjusted to the same level (0·33 U/ml, see Methods). However, cellobiase activity of *Penicillium* cellulase was 47 times higher than *T. reesei* (PFP) and 3·6 times higher than *T. reesei* (Genencor) (Table 3).

Penicillium cellulase demonstrated the best parameters of MCC hydrolysis. Initial rate of glucose formation was 10·4 g/l-h, concentration of glucose in 24 h hydrolysate was 41 g/l or 91% of total sugars. *Trichoderma* cellulases demonstrated lower efficiency. In 24 h concentration of glucose reached only 15 g/l for *T. reesei* (PFP) and 26 g/l for *T. reesei* (Genencor). Moreover, *Trichoderma* hydrolysates contained 24–40% cellobiose compared to a much lower percentage of cellobiose in *Penicillium* hydrolysate (9%). These results can be explained by the insufficient cellobiase activity in *Trichoderma* preparations (Table 1). Addition of cellobiase from *Aspergillus* preparations had a very favorable effect on the rate and extent of MCC hydrolysis. Initial rate of glucose formation increased approximately five times (compare #2 and #3, Table 3), final glucose concentration increased to 32 (#3) and 37 g/l (#5) and the content of cellobiose in 24 h hydrolysate decreased to the level of *Penicillium* hydrolysate. However, the initial rate of glucose formation and final glucose concentration have not reached the values obtained with *Penicillium* cellulase. This could be explained by a more balanced composition of the enzyme system produced by *Penicillium* sp. or by better catalytic properties of its individual cellulolytic enzymes. For example, *Penicillium* enzymes could be more resistant to end-product inhibition. To confirm these suggestions further work on isolation and purification of individual enzymes from *Penicillium* sp. and investigation of their properties should be performed.

Superior properties of the *Penicillium* cellulase can be also explained by the higher operational stability of its enzymes. Table 4 summarizes the data on inactivation of the preparations studied more during incubation at pH 4·5 and 50°C with or without stirring. In 24 h incubation under stirring, FPA of *Penicillium* cellulase decreased to 92%, *T. reesei* (PFP) to 30% and *T. reesei* (Genencor) to 42%. FPA of a combined preparation of *T. reesei* (PFP) and *A. foetidus* (PFP) decreased to 28%, *T. reesei* (Genencor) and *A. niger* (Novo) decreased to 44%. Contribution of stirring to FPA inactivation was only

3% for *Penicillium* cellulases, while it was 10–35% for the other cellulases.

Therefore, at least two factors — higher level of cellobiase activity and higher operational stability of *Penicillium* cellulase — provide more effective hydrolysis of MCC by this preparation compared to *T. reesei* preparations.

CONCLUSIONS

Composition, stability at different pH and temperatures and hydrolytic effciency toward MCC of *Penicillium* cellulase were evaluated compared to commercial *Trichoderma reesei* cellulases and combined preparations consisting of *Trichoderma* and *Aspergillus* cellulases.

For *Penicillium* cellulase the pH optimum of FPA was 4·5–5·0 and temperature optimum 50–55°C. Maximum stability of FPA was observed at pH 4·0–5·0 and temperature 40–50°C. Stirring did not significantly affect FPA and cellobiase activity of *Penicillium* preparation. The contribution of stirring in inactivation was only 3–4%.

Kinetic parameters of MCC hydrolysis by *Penicillium* cellulase were determined at optimal conditions (pH 4·5, 50°C): K_m=50 g/l, V_{max}=330 μM/min per gram of protein.

Penicillium preparations demonstrated more effective saccharification of MCC compared to *Trichoderma reesei* cellulases with the same level of total cellulolytic activity (1·0 FPU/ml). Glucose concentration in 24 h hydrolysate was 41 g/l for *Penicillium* cellulase, 15 g/l for *T. reesei* (PFP) and 26 g/l for *T. reesei* (Genencor). At the same time the hydrolysate obtained with *Penicillium* cellulase contained only 9% of cellobiose compared to 24–40% cellobiose in the case of *Trichoderma reesei* cellulases. It was shown that at least two factors — the higher level of cellobiase activity in *Penicillium* preparations and its higher stability at operational conditions — can explain the superior properties of this cellulase compared to *Trichoderma reesei* cellulases.

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WASTE BIOGAS RESIDUAL SLURRY AS AN ADSORBENT FOR THE REMOVAL OF Pb(II) FROM AQUEOUS SOLUTION AND RADIATOR MANUFACTURING INDUSTRY WASTEWATER

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Abstract

Waste biogas residual slurry (BRS) was used for the adsorption of Pb(II) from aqueous solution, over a range of initial metal ion concentrations (20–100 mg litre^{-1}), agitation times (5–70 min), adsorbent doses (0·4–5·0 g litre^{-1}) and initial pH values (1·5–6·0). The amount of Pb(II) adsorbed (mg g^{-1} of adsorbent) increased with increases in the initial concentration of Pb(II). The applicability of the Lagergren rate equation was also investigated. The process of uptake of Pb(II) by BRS followed the Langmuir isotherm model and the adsorption capacity was 28 mg g^{-1}. An almost quantitative removal of Pb(II) from a solution (50 ml) containing 100 mg litre^{-1} Pb(II) by 5·0 g litre^{-1} adsorbent was observed at an initial pH of 2·5. Application of BRS for the effective removal of Pb(II) from radiator manufacturing industry wastewater has been demonstrated.

Key words: Biogas residual slurry, Pb(II), adsorption, wastewater.

INTRODUCTION

The presence of heavy metals in the environment is of major concern because of their toxicity and threat to human life and the environment. Lead is a heavy-metal poison which, acting by complexing with oxo-groups in enzymes, virtually affects all steps in the process of haem synthesis and porphyrin metabolism (Greenwood & Earnshaw, 1989).

Anthropogenic sources of heavy metals include wastes from the electroplating and metal-finishing industries, metallurgical industry, tannery operations, chemical manufacturing, mine drainage, battery manufacturing, leachates from landfills and contaminated ground water from hazardous waste sites.

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For wastes with high metal concentrations, precipitation processes (e.g. hydroxide, sulphide) are the most economical. However, many metal-bearing wastes contain substances, such as complexing agents, that decrease the effectiveness of precipitation processes and lead to relatively high metal concentrations in the effluent. In addition, the current regulatory trend is for heavy-metal discharge limits approaching those of drinking water standards. Thus, additional treatment processes are required to 'polish' the effluent prior to discharge.

Activated carbon has not been widely used for removal of heavy metals from water, but a considerable amount of research has been conducted on the subject (Netzer & Hughes, 1984; Koshima & Onishi, 1986; Corapcioglu & Huang, 1987). A number of low-cost absorbents have been reported in the literature for the removal of Pb(II), such as fly ash (Mathur & Rupainwar, 1988), bottom ash from thermal power plants (Kaur *et al.*, 1991), steel-plant granulated slag (Loomba & Panday, 1993), oil-palm fibre and coconut husk (Latif & Jaafar, 1989), coconut-shell carbon (Arulananthan *et al.*, 1989), peanut-hull carbon (Periasamy & Namasivayam, 1994), onion skin polymerized with formaldehyde (Kumar & Dara, 1981), EDTA-modified groundnut husk (Okieimen *et al.*, 1991), EDTA-modified cellulosic materials (Okieimen & Onyenkba, 1989*a*), melon seed husks (Okieimen & Onyenkba, 1989*b*) and thiolated maize-cob meal (Okieimen *et al.*, 1989), *Calymperes delessertii besch* (Low & Lee, 1987) and peat moss (Coupal & Lalancette, 1976). Goethite (Forbes *et al.*, 1976), ferric oxide (Aualitia & Pickering, 1987), waste Fe(III)/Cr(III) hydroxide (Namasivayam & Ranganathan, 1994) have also been tried for the removal of lead from wastewaters.

Biogas residual slurry, a lignocellulosic waste, is a by-product of biogas plants. Because of the government subsidy for the installation of biogas plants in India, a vast number of biogas plants have been built

in individual houses in the villages. Hence, the availability of biogas residual slurry is great. We have recently reported the application of biogas residual slurry for the removal of the dyes congo red (Namasivayam & Yamuna, 1992*a*), rhodamine-B (Namasivayam & Yamuna, 1992*a*), acid brilliant blue (Yamuna & Namasivayam, 1993) and direct red 12-B (Namasivayam & Yamuna, 1994) from wastewaters.

The aim of this study was to investigate the feasibility of using biogas residual slurry (BRS) as an absorbent for the removal of Pb(II) from aqueous solutions and radiator manufacturing industry wastewater. Parameters which could influence the removal, such as Pb(II) concentration, agitation time, pH and adsorbent dose were studied.

METHODS

The adsorbent was collected from the outlet of a biogas plant which was fed only with cow dung. The fermented slurry was dried in sunlight and the cakes formed were powdered using a stone-grinder. The powder was sieved to give particle size ranges of 53–75, 75–150 and ≥ 500 μm.

For the characterization of BRS slurry, the adsorbent with particle size 75–150 μm was used. pH_{ZPC} (Kinniburg *et al.*, 1975), surface area (Shoemaker & Garland, 1967) and porosity (calculated from bulk and particle density values, previously determined by core and pycnometer method; Blake & Hartge, 1986) were estimated according to published procedures. Moisture content was determined gravimetrically and electrical conductivity was measured in 2% solution (Wilde *et al.*, 1972). Sodium, potassium and calcium were determined using a flame photometer and carbon and sulphur by LECO elemental analyser. The characteristics of BRS are shown in Table 1.

All the chemicals used were of analytical reagent grade. A stock solution of 1000 mg litre^{-1} Pb(II) was prepared from $Pb(NO_3)_2$ in double distilled water containing a few drops of concentrated HNO_3 to prevent hydrolysis. This solution was diluted as required to obtain standard solutions containing 20–100 mg litre^{-1} Pb(II). Fifty millilitres of Pb(II) solution of a desired concentration and adjusted to pH 2·5 was taken in 100 ml capacity conical flasks containing known amounts of adsorbent.

Table 1. Characteristics of biogas resident slurry

Characteristic	Result
pH_{ZPC}	8·9
Surface area (m^2 g^{-1})	160
Bulk density	0·74
Specific gravity	1·86
Porosity (%)	60·17
Moisture content (%)	3·525
Ash content (%)	54·93
Conductivity (μs cm^{-1})	426
Ash analysis	
Potassium (%)	0·75
Calcium (%)	0·70
Sodium (%)	0·17
Carbon (%)	0·50
Sulphur (%)	0·23

Batch adsorption studies were conducted by agitating the flasks for a predetermined period at 30 ± 1°C using a shaking water bath. The adsorbent was separated by centrifugation at 14000 rev. min^{-1} and the supernatant was analysed for pH and Pb(II). The residual concentration of Pb(II) was determined spectrophotometrically using 4-(2-pyridylazo resorcinol) (Pollard *et al.*, 1959). The Langmuir adsorption isotherm study was carried out with different initial concentrations of Pb(II) from 20 to 100 mg litre^{-1} while maintaining the adsorbent dose at 2·0 g litre^{-1}. The effect of adsorbent dose was studied using 50 ml solution of 100 mg litre^{-1} Pb(II) and varying amounts of BRS from 20 to 250 mg. The pH effect was studied using 100 mg of adsorbent and 50 ml solution of 100 mg litre^{-1} Pb(II). Sodium, potassium and calcium in solutions were estimated using a flame photometer.

Desorption studies were carried out as follows: after adsorption experiments with 100 mg litre^{-1} Pb(II) and 200 mg of BRS, the lead-laden adsorbent samples containing 3·8 mg of Pb(II) were separated and gently washed with distilled water to remove any unadsorbed Pb(II). Several such spent adsorbent samples were prepared. They were then agitated with 50 ml of distilled water adjusted to various acidic pH values using dilute HNO_3 and the desorbed lead was estimated by analysing the acid solutions as before.

Industrial wastewater containing Pb(II) was collected from a radiator-manufacturing industry in Coimbatore city. Since the Pb(II) concentration was very high in the wastewater, it was diluted to give a concentration of 120 mg litre^{-1}. Effects of pH and adsorbent dose on removal of Pb(II) were examined using 50 ml aliquots of wastewater. The adsorption capacity of BRS for the removal of Pb(II) from the industrial wastewater was determined from the Langmuir isotherm using Pb(II) concentrations varying from 24 to 120 mg litre^{-1} and an adsorbent dose of 2·0 g litre^{-1}.

All the experiments were performed in duplicate and mean values are presented. Maximum deviation was 3%.

RESULTS AND DISCUSSION

Effect of agitation time and initial concentration

Figure 1 shows the effect of agitation time on the removal of Pb(II) by BRS. The removal (mg g^{-1}) increases with time and attains equilibrium at 30 min for an initial Pb(II) concentration of 20, 50, 75 or 100 mg litre^{-1}. The plots are single, smooth and

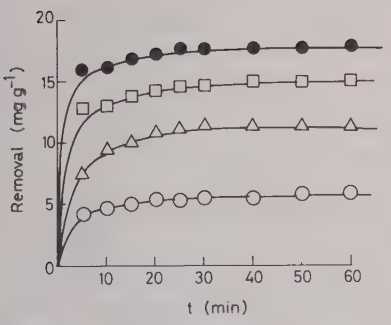

Fig. 1. Effect of agitation time on the adsorption of Pb(II). Pb(II) concentration: (○) 20 mg litre^{-1}, (△) 50 mg litre^{-1}, (□) 75 mg litre^{-1}, (●) 100 mg litre^{-1}, BRS dose: 2·0 g litre^{-1}; particle size: 75–150 μm; initial pH: 2·5.,

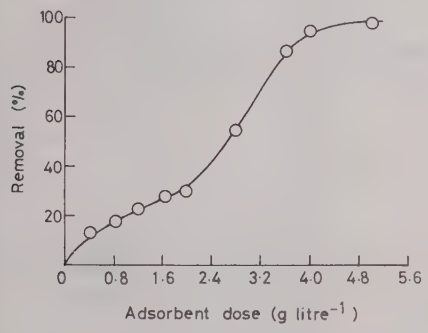

Fig. 2. Effect of adsorbent dosage on the adsorption of Pb(II) concentration: 100 mg litre^{-1}; particle size 75–100 μm; initial pH: 2·5.

continuous, leading to saturation, suggesting the possibility of the formation of monolayer coverage of Pb(II) on the surface of BRS (Namasivayam & Kanchana, 1992).

Effect of adsorbent dose

Figure 2 shows the removal of Pb(II) as a function of BRS dosage at different initial pH values. It is evident that for the quantitative removal of Pb(II) from a 50 ml solution of 100 mg litre^{-1} a minimum BRS dose of 250 mg is required at an initial pH of 2·5.

Effect of contact time and particle size

Figure 3 shows a series of contact time curves for adsorbent particle size ranging from 75–150 μm to >500 μm. Removal of Pb(II) (mg g^{-1}) increases from 21·00 to 35·00 with a decrease in particle size of BRS from >500 to 75–150 μm. This indicates that the smaller the adsorbent particle size, and hence the larger the surface area, the greater will be the adsorption.

Adsorption kinetics

The kinetics of Pb(II) adsorption on BRS follow the first-order rate expression given by Lagergren (Namasivayam & Yamuna, 1992*a*):

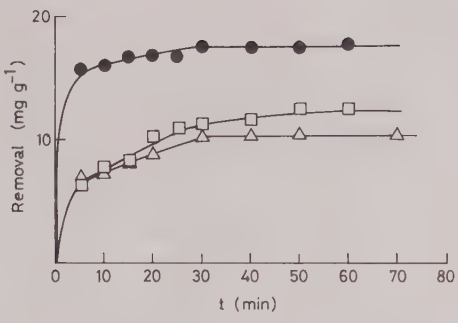

Fig. 3. Effect of agitation time and particle size on the adsorption of Pb(II). Pb(II) concentration: 100 mg litre^{-1} particle size: (●) 75–150 μm, (□) 250–500 μm, (△) >500 μm; BRS dose: 2·00 g litre^{-1}; initial pH: 2·5.

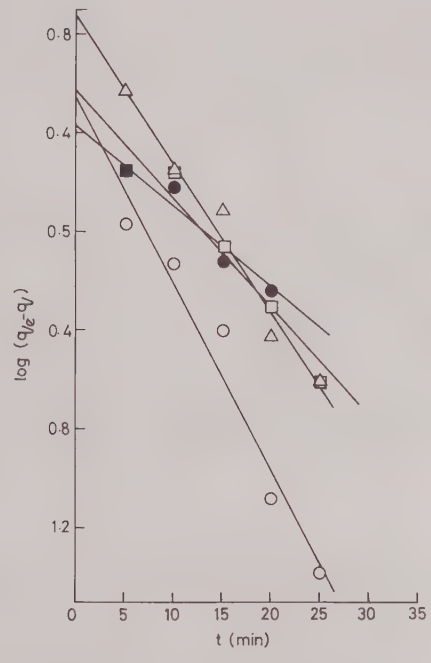

Fig. 4. Lagergren plot for the adsorption of Pb(II). Pb(II) concentration: (○) 20 mg litre^{-1}, (△) 50 mg litre^{-1}, (□) 75 mg litre^{-1}, (●) 100 mg litre^{-1}; BRS dose: 2·0 g litre^{-1}; particle size 75–150 μm; initial pH: 2·5.

$$\log(q_e - q) = \log q_e - \frac{k_{ad} t}{2{\cdot}303} \qquad (1)$$

where q is the amount of Pb(II) adsorbed (mg g^{-1}) at time t, q_e is the amount adsorbed (mg g^{-1}) at equilibrium time and k_{ad} is the rate constant of adsorption (min^{-1}). Linear plots of log ($q_e - q$) vs t show the applicability of the above equation to different initial Pb(II) concentrations and adsorbent particle-size ranges (Figs 4 and 5). The k_{ad} values for different initial Pb(II) concentrations and particle-size ranges are shown in Table 2. Increasing the Pb(II) concentration and lowering the adsorbent particle size increase the k_{ad} value. Similar results have been reported for the adsorption of Pb(II) by

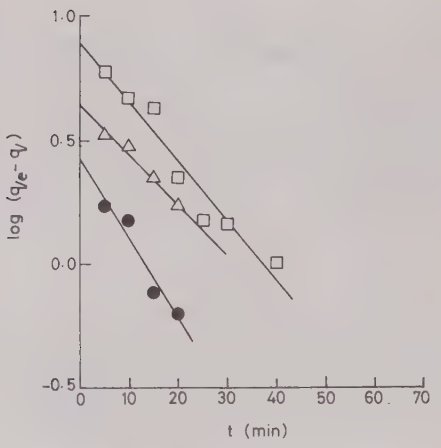

Fig. 5. Lagergren plot for the adsorption of Pb(II). Pb(II) concentration: 100 mg litre $^{-1}$; particle size: (●) 75–150 μm, (□) 250–500 μm, (△) >500 μm; BRS dose: 2·00 g litre $^{-1}$; initial pH: 2·5.

Table 2. Adsorption rate constants[a]

Pb(II) concentration (mg litre^{-1})	Rate constant[b] k_{ad} (min^{-1})	Particle size (μm)	Rate constant[c] k_{ad} (min^{-1})
20	$1{\cdot}75\times10^{-1}$	75–150	$7{\cdot}56\times10^{-2}$
50	$1{\cdot}39\times10^{-1}$	250–500	$5{\cdot}58\times10^{-2}$
75	$1{\cdot}03\times10^{-1}$	>500	$4{\cdot}52\times10^{-2}$
100	$7{\cdot}56\times10^{-2}$		

[a] Conditions: adsorbent dose, 2·0 g litre^{-1}; initial pH, 2·5; [b] particle size, 75–150 μm; [c] Pb(III) concentration, 100 mg litre^{-1}.

coconut-shell carbon and granular activated-carbon (Arulanantham *et al.*, 1989).

Adsorption isotherm

The Langmuir equation was applied for adsorption equilibrium (Namasivayam & Périasamy, 1993).

$$C_e/q_e = 1/(Q_o b) + C_e/Q_o \qquad (2)$$

where C_e is the equilibrium concentration of adsorbate (mg litre^{-1}), q_e is the amount adsorbed at equilibrium (mg g^{-1}) and Q_o and b are Langmuir constants related to adsorption capacity and energy of adsorption, respectively. The linear plot of C_e/q_e vs C_e shows that the adsorption follows the Langmuir isotherm model (Fig. 6). Constants Q_o and b were determined from Langmuir plots and are presented in Table 3.

The essential characteristics of the Langmuir isotherm can be expressed in terms of a dimensionless constant separation factor or equilibrium parameter, R_L, which is defined by

$$R_L = \frac{1}{1 + bC_o} \qquad (3)$$

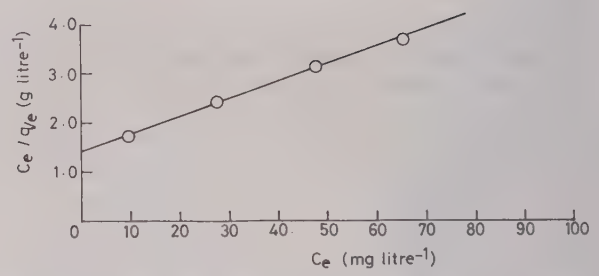

Fig. 6. Langmuir plot for the adsorption of Pb(II). Pb(II) concentration: 20–100 mg litre $^{-1}$; BRS dose: 2·0 g litre^{-1}; initial pH: 2·5.

Table 3. Langmuir isotherm data

Q_o (mg g^{-1})	b (litre mg^{-1})	Initial Pb(II) concentration (mg litre^{-1})	R_L
27·9	0·025	20	$4{\cdot}88\times10^{-2}$
		50	$1{\cdot}95\times10^{-2}$
		75	$1{\cdot}30\times10^{-2}$
		100	$9{\cdot}76\times10^{-3}$

where b is the Langmuir constant and C_o is the initial concentration of Pb(II) (Yamuna & Namasivayam, 1993). The R_L values indicate the type of isotherm. According to McKay *et al.* (1980):

R_L value	Type of isotherm
$R_L>1$	Unfavourable
$R_L=1$	Linear
$0<R_L<1$	Favourable
$R_L=0$	Irreversible

R_L values between 0 and 1 indicate favourable adsorption of Pb(II) on BRS for the Pb(II) concentrations studied at 30±1°C (Table 3).

Effect of pH

The conventional method for the removal of heavy metals is by precipitation as hydroxides or sulphides. This method has the limitation that metals cannot be completely removed from solution owing to the solubility product of metal hydroxides or sulphides. Hence, comparison is made between adsorption and precipitation as metal hydroxide. Effects of pH on Pb(II) removal in the presence and absence of adsorbent are shown in Figs 7 and 8 for different concentrations of Pb(II). Figure 7 also presents percent adsorption vs final pH. The final pH is always greater than the initial pH except for very low pH values, where a buffering effect occurs. It is evident that adsorption is much more efficient than metal hydroxide precipitation in the absence of adsorbent. For a Pb(II) concentration of 100 mg litre^{-1}, adsorption starts at a pH as low as 1·5 and increases with an increase in pH and attains a maximum of 95% at an initial pH≥3·5, where precipitation of

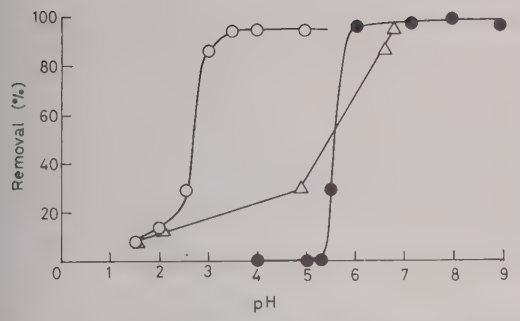

Fig. 7. Effect of pH on the removal of Pb(II). Pb(II) concentration: 100 mg litre^{-1}; BRS dose 2·0 g litre^{-1}; particle size: 75–150 μm; (○) percent removal vs initial pH; (△) percent removal vs final pH; (●) hydroxide precipitation.

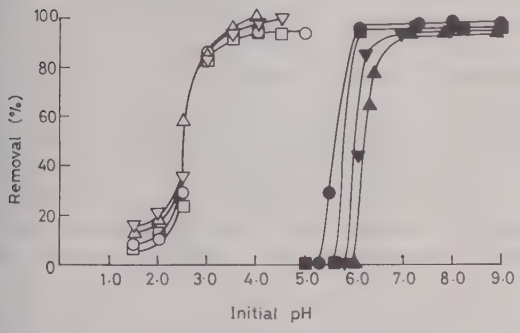

Fig. 8. Effect of pH on the removal of Pb(II). Pb(II) concentration: (△) 20 mg litre^{-1}, (▽) 40 mg litre^{-1}, (□) 80 mg litre^{-1}, (○) 100 mg litre^{-1}; BRS dose: 2·0 g litre^{-1}; (▲, ▼, ■, ●) corresponding hydroxide precipitation.

Pb(II) as hydroxide does not even start. Precipitation starts only at pH 5·5 and attains 95% at pH 6·0. The influence of pH on Pb(II) removal can be explained on the basis of an electrostatic interaction model (Bhattacharya & Venkobachar, 1984). As the pH increases, the number of positively-charged sites on the adsorbent surface decreases and the electrostatic attraction of positively-charged lead species, Pb^{2+} and $PbNO_3^+$, is favoured (Herbert *et al.*, 1953).

The major mechanism of adsorption of Pb(II) seems to be ion exchange. When oxo groups (C_xO and C_xO_2), present on the surface of carbonaceous materials, come into contact with water they hydrolyse water molecules as shown below (Sharma & Forster, 1994).

$$C_xO + 2H_2O \rightarrow C_xOH_2^{2+} + 2OH^- \quad (4)$$

Thus, the hydroxyl ions released into solution raise the equilibrium pH. Since BRS contains Na^+, K^+ and Ca^{2+} ions, groups such as $C_xOX_2^{2+}$, C_xOCa^{2+} are also assumed to be present (where x=Na^+ or K^+). Alkali metal ions in the above groups are also exchanged with H^+ in the medium as follows:

$$C_xOX_2^{2+} + 2H^+ \rightarrow C_xOH_2^{2+} + 2X \quad (5)$$

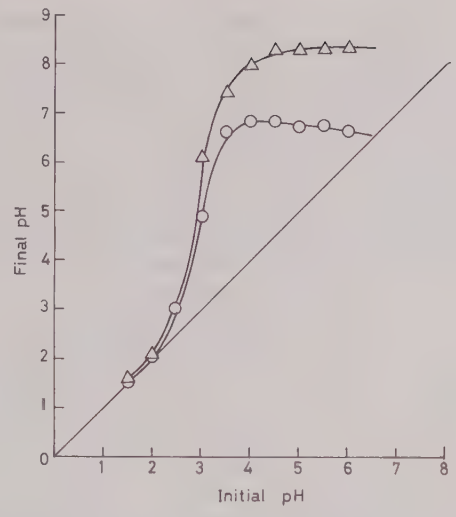

Fig. 9. Effect of initial pH on final pH in the presence and absence of Pb(II). Pb(II) concentration: (○) 100 mg litre^{-1}; (△) 0 mg litre^{-1}; BRS dose: 2·0 g litre^{-1}; particle size: 75–150 μm.

$$C_xOCa^{2+} + 2H^+ \rightarrow C_xOH^{+2} + Ca^{2+} \quad (6)$$

Reactions (4)–(6) contribute to an increase in pH. The experiment with distilled water (blank) exhibited such an increase in pH (Fig. 9). At the same time reactions (5) and (6) lead to the release of Na^+, K^+ and Ca^{2+}. When Pb(II) is present in solution, its adsorption will free some H^+ and the eventual pH increase will be lower than in the blank (eqn (7)) (Fig. 9)

$$C_xOH_2^{2+} + Pb^{2+} \rightarrow C_xOPb^{2+} + 2H^+ \quad (7)$$

At the same time Na^+ and Ca^{2+} will also be released according to reactions:

$$C_xONa_2^{2+} + Pb^{2+} \rightarrow C_xOPb^{2+} + 2Na^+ \quad (8)$$

$$C_xOCa^{2+} + Pb^{2+} \rightarrow C_xOPb^{2+} + Ca^{2+} \quad (9)$$

Figure 9 shows the effect of initial pH on final pH. The curve referred to as blank was obtained under conditions such that [Pb(II)]=0. A test performed by agitating 100 mg BRS for 1 h at an initial pH of 2·5 led to a sodium concentration of 2·8 mg litre^{-1} (blank) in the aqueous solution. Also, when the Pb(II) concentration of 100 mg litre^{-1} was used in the treatment, the release of Na^+ was found to increase from 2·8 (blank) to 5·2 mg litre^{-1} and Ca^{2+} from 13·3 (blank) to 15·8 mg litre^{-1}. There was no change in the concentration of the released K^+ ion between the blank and Pb(II) sample. The observations indicate that apart from exchange of H^+ ions on the adsorbent with Pb(II) ions, significant Na^+ and Ca^{2+} ions, which were present in the adsorbent, were also exchanged with Pb(II) ions (Table 1).

Desorption studies

Desorption studies help elucidate the adsorption mechanism. Also they aid recovery of precious met-

als from wastewaters and recycle the adsorbent. Desorption of Pb(II) from the spent BRS was carried out using dilute HNO_3. The percent recovery of Pb(II) was found to be 33·8, 68·2 and 79·8 at pH 2·00, 1·50 and 1·00, respectively. At acidic conditions, protonation of the adsorbent surface replaces the metal ions on the adsorbent surface leading to the desorption of the positively-charged metal ions. This is further evidence that ion-exchange is involved in the adsorption mechanism.

Tests with radiator-manufacturing industry wastewater

The characteristics of the radiator-manufacturing industry wastewater are given in Table 4.

Effect of adsorbent dose on Pb(II) removal

Figure 10 shows the effect of adsorbent dose on the removal of Pb(II) from the industrial wastewater containing 120 mg litre^{-1} Pb(II) at an initial pH of 2·5. A maximum removal of 83% was obtained at a BRS dose of 6·0 g litre^{-1}.

Effect of pH on removal of Pb(II)

Figure 11 shows the effect of pH on Pb(II) removal from the industrial wastewater containing 120 mg litre^{-1} Pb(II). A maximum removal of 90% by 2·0 g litre^{-1} was attained at an initial pH ≥ 4·5.

Table 4. Characteristics of radiator-manufacturing industry wastewater

Characteristic	Result
pH	1·63
Conductivity (mS cm^{-1})	14·43
Turbidity (NTU)	45
Total suspended solids (g litre^{-1})	32
Total dissolved solids (g litre^{-1})	435
Hardness (mg litre^{-1})	6834
Sulphate (mg litre^{-1})	63·0
Chloride (mg litre^{-1})	2521
Sodium (mg litre^{-1})	215
Potassium (mg litre^{-1})	18·4
Calcium (mg litre^{-1})	225
Lead (mg litre^{-1})	551

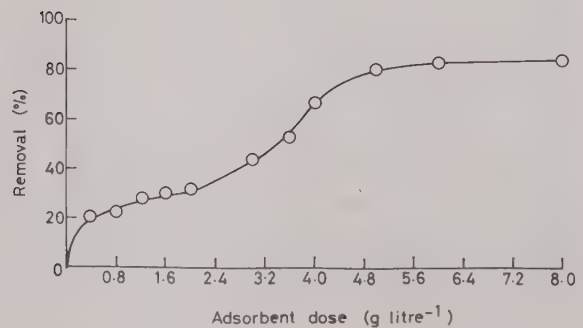

Fig. 10. Effect of adsorbent dose on Pb(II) from radiator-manufacturing industry wastewater. Pb(II) concentration: 120 mg litre^{-1}; particle size: 75–150 μm; initial pH: 2·5.

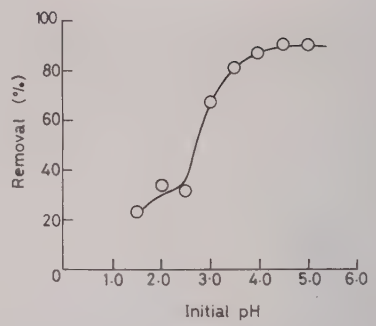

Fig. 11. Effect of pH on the removal of Pb(II) from radiator-manufacturing industry wastewater. Pb(II) concentration: 120 mg litre^{-1}; BRS dose: 2·0 g litre^{-1}; particle size: 75–150 μm.

Adsorption capacity of BRS

The adsorption capacity (Q_o) of BRS for the removal of Pb(II) from the industrial wastewater, obtained from the Langmuir plot of C_e/q_e vs C_e was 28·6 mg g^{-1} (plot not shown). This shows that the adsorption capacity of BRS for Pb(II) is not affected by the other components present in the industrial wastewater.

CONCLUSION

The present study shows that biogas residual slurry is an effective adsorbent for removal of Pb(II) from aqueous solution. Adsorption followed the Langmuir isotherm and the adsorption capacity was 27·9 mg g^{-1}. The data may be helpful for designing and fabricating a wastewater-treatment plant for the removal of Pb(II). Application of BRS for the effective removal of Pb(II) from radiator-manufacturing industry wastewater has also been demonstrated. The aqueous extract of BRS still contains macro- and micronutrients and hence the treated wastewater containing an aqueous extract of BRS may be directly utilized for irrigational purposes.

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MICROBIOLOGICAL AND BIOCHEMICAL CHANGES DURING THE COMPOSTING OF OIL PALM EMPTY-FRUIT-BUNCHES. EFFECT OF NITROGEN SUPPLEMENTATION ON THE SUBSTRATE

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Abstract

The composting of oil palm empty-fruit-bunches and of oil palm empty-fruit-bunches in supplementation with either goat dung, cow dung or chicken manure differed in the resulting C:N ratios. The initial C:N ratios (52:1, 35:1, 48:1, 47:1) for the four compost heaps were significantly reduced to 24:1, 14:1, 18:1 and 12:1, respectively, after 60 days of composting, resulting in the production of a stable humus that is suitable for crop production. A temperature of 70°C was maintained for 3 days at the onset of composting. Both mesophilic and thermophilic bacteria showed consistent activity throughout the process, whereas fungal activity was completely suppressed during the peak heating phase. The rate of utilization of cellulosic material showed a positive correlation with the increase in the nitrogen content of the compost.

Key words: Oil palms, empty-fruit-bunches, manure additions, compost, cellulose, carbon:nitrogen, microorganisms.

INTRODUCTION

The Malaysian palm oil industry generated 1·16 million tonnes of empty-fruit-bunches (EFB) in 1985 alone, the volume predicted to increase to 2·16 million tonnes by 2000 (Ministry of Primary Industries, Malaysia, 1991). Mulching currently accounts for only a fraction of the EFB that are discarded; these are normally burnt in incinerators for the ash as fertilizer. Currently there is much interest in utilizing palm oil waste in general (Thambirajah & Kuthubutheen, 1989). Composting has been suggested as an alternative to incineration of the waste as the process converts the waste, which is essentially organic in nature, into a humus that is suitable for crop production (Gomez & Park, 1983; Gray *et al.*, 1971). In composting, the higher-plant material breaks down under the influence of aerobic thermophilic microorganisms present in the waste to a material rich in organic nutrients.

On one hand, the EFB component of palm-oil waste presents particular difficulties both in its transportation and in its incineration, owing to its extensive bulk. On the other hand, EFB contain a high proportion of cellulosic matter which is easily decomposed by a combination of physical, chemical and biological processes. The bunch consists of 70% moisture and 30% solids; of which holocellulose accounts for 65·5%, lignin 21·2%, ash 3·5%, hot-water-soluble substances 5·6% and alcohol–benzene solubles 4·1% (Husin *et al.*, 1985). In an extension of an earlier study (Thambirajah & Kuthubutheen, 1989), on the composting of palm press fibre, we report in this paper the results of a similar study on EFB.

METHODS

Materials

Oil palm empty-fruit-bunches were shredded into loose fibrous material by using a shredder manufactured by B & W Engineering, Angel Drove, Ely, Camb. (Serial No. R/N 30/12). Goat dung, cow dung and chicken manure (poultry broiler floor litter) were collected from the research farm of the Institute of Advanced Studies.

Compost

Four heaps were prepared as follows: the first (Heap 1) had 90 kg EFB, the second (Heap 2) 90 kg EFB and 25 kg goat dung, the third (Heap 3) 90 kg EFB and 25 kg cow dung and the fourth (Heap 4) 90 kg EFB and 25 kg chicken manure (broiler floor litter). Water was added to each of the heaps to a final moisture content of 65% w/w. The heaps were turned once every week for 8 weeks. However, during the first week the heaps were turned on the third

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day as well, when the temperature in the heaps rose rapidly.

Sampling
Grab samples (approx. 200 g) were collected from the interior of the heaps that were turned weekly. The samples were bulked and portioned into two lots. Microbial enumeration was performed on one lot. The other was dried to constant weight (60°C for 2 days) for the chemical analyses. Microbial counts were calculated on the basis of dry-matter weight.

Temperature
The temperature at the top, middle and bottom layers of the interior of the heaps was read daily at 09.30 by using a thermocouple thermometer (Suntex ST-52K type). Ambient temperature was also recorded daily throughout the process.

Chemical analysis
Carbon contents were determined on dried, finely-ground (1 mm) samples by using the method of Tinsley (Allen *et al.*, 1974). The cellulose content of the fibres was determined by using the method of Updegraff (1969). Total nitrogen was determined by using the Kjeldahl method (AOAC, 1975). Changes in lignin content were followed by using the method of Zadrazil and Brunnert (1980). Changes in pH were monitored by using 10 g of dried, finely-ground samples suspended in 50 ml distilled water.

Microbial counts
The dilution plate method was used to enumerate the microorganisms in the compost heaps. A sample (10 g) from the grab lot, together with sterile distilled water (90 ml), was homogenized in a stomacher (model SF 400) for 2 min. Serial dilutions of this stock were made and used to inoculate plates in triplicate (Cappuccino & Sherman, 1983). Nutrient agar, corn meal agar, yeast extract agar and actinomycete agar were used in the counting of the number of bacteria, mesophilic fungi, thermophilic fungi and actinomycetes, respectively. The plates were incubated at 35 and 45°C for mesophilic and thermophilic bacterial counts and at 30 and 45°C for the mesophilic and thermophilic fungal and actinomycetic counts. Bacterial counts were recorded 48 h after plating, whereas fungal and actinomycetic counts were made 72 h after plating.

Statistical analysis
Statistical analyses of the data were performed by using the subroutine of the Statgraphics package (Statistical Graphics Corporation, 1987), with response variables being C:N ratio, carbon concentration, nitrogen levels, cellulose, lignin and pH. The Multifactor Analysis of Variance Program gave the effect of each treatment on the response variables with the day of collection as a co-variant.

RESULTS AND DISCUSSION

The composting of EFB resulted in a significant reduction in the C:N ratios when manure was included in the substrate. This correlated with the microbial activity which was evident from the increases in the temperature of the heaps. In composting, the C:N ratio indicates the maturity of the product (Gray *et al.*, 1971; Chanyasak & Kubota, 1981; Jimenez & Garcia, 1989). An initial ratio ranging between 30:1 and 50:1 that converges to a final value of 10:1 to 15:1 is often taken as a measure of a stable humus (Taiganides, 1977). The ratio for most agricultural waste is often as high as 70:1, but can be lowered to a more appropriate level by the addition of nitrogenous supplements such as manure.

The initial C:N ratios of Heap 1 (control), Heap 2 (EFB and goat dung), Heap 3 (EFB and cow dung) and Heap 4 (EFB and chicken manure) (52:1, 35:1, 48:1 and 47:1, respectively) were reduced to 24:1, 14:1, 18:1 and 12:1 after 60 days (Fig. 1). These values were interpreted in terms of a mature compost and they approximated the limiting ranges of 15:1 to 20:1 (Poincelot, 1974). Supplementation with chicken manure afforded a compost with the lowest ratio when the proportion of EFB to manure was fixed at 3:1.

The number of mesophilic and thermophilic microorganisms fluctuated with respect to changes in temperature of the heaps; higher temperatures can destroy pathogenic microorganisms and other plant material. The temperature of the control (Heap 1) increased rapidly to about 75°C within 1 day and then dropped gradually to 40°C after 21 days, after which time the heap slowly attained ambient temperature (Fig. 2). This trend was also observed for the heaps supplemented with the dung (Figs 3–5) but the three exhibited a higher overall temperature increase for the same period. Microbial counts (Figs 6–11) were higher for these heaps. A maximum temperature of 75°C appears sufficient to effect the destruction of pathogens (Spaggiari & Spigoni, 1986).

Three main groups of microorganisms — bacteria, fungi and actinomycetes — determine the biodegradation pattern of cellulosic plant material (Fergus, 1964; Chang & Hudson, 1967) and are responsible for the physical and chemical changes during composting. In this study on EFB, the bacterial and actinomycetic counts were low during the peak heating phase, but increased subsequently, remaining without change for the duration of the process. However, fungi appeared to be completely absent during the peak heating phase (Figs 8 and 9), an observation similar to that made on wheat straw compost (Chang & Hudson, 1967).

Depending on the conditions of growth, the substrate and the organisms involved, the end products

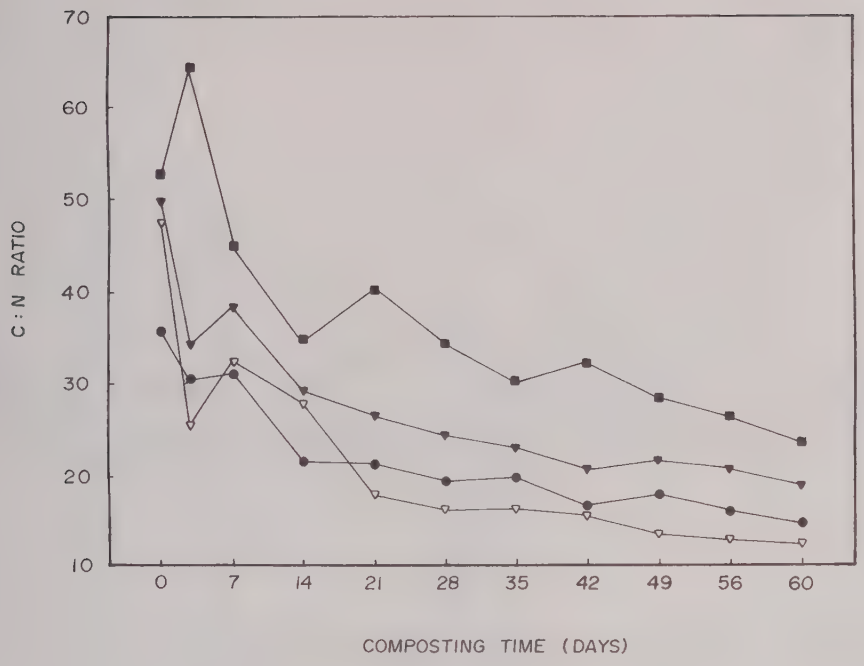

Fig. 1. Carbon versus nitrogen plot of the four heaps. ■, Heap 1; ●, Heap 2; ▼, Heap 3; ▽, Heap 4.

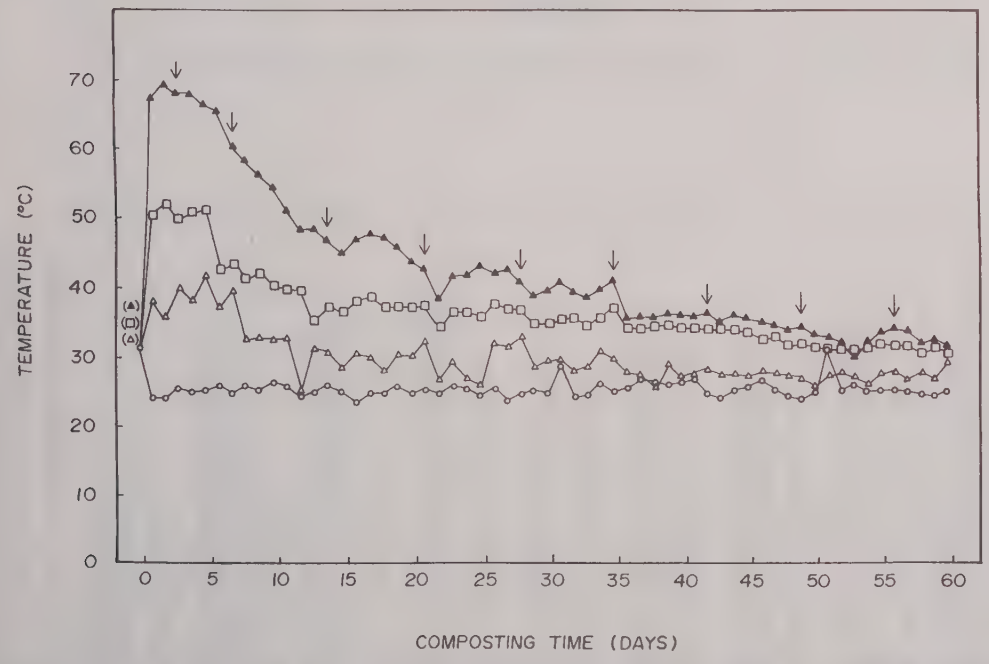

Fig. 2. Temperature–time plot of the top △, middle ▲ and bottom □ layers of Heap 1. ○ Indicates ambient temperature. Arrows (↓) indicate turning intervals of the heap.

of fermentation vary greatly. Microbial fermentation of carbohydrates generally results in an increase in acidity (Garg & Neelakantan, 1982). *Clostridium* species commonly ferment glucose to yield butyl and other alcohols and certain acids. *Lactobacillus lactis* yields almost entirely lactic acid, while *Lactobacillus brevis* yields lactic and acetic acids, ethyl alcohol and carbon dioxide (Frobisher *et al*., 1974).

In composting, carbohydrates are also broken down to humic and fulvic acids (Spaggiari & Spigoni, 1986). However, the fulvic acid is subsequently degraded. This, together with ammonification of inorganic nitrogen, accounts for the neutral pH which is generally attained at the end of the process (Chang & Hudson, 1967; MacGregor *et al*., 1981). The initial pH levels in the present study averaged

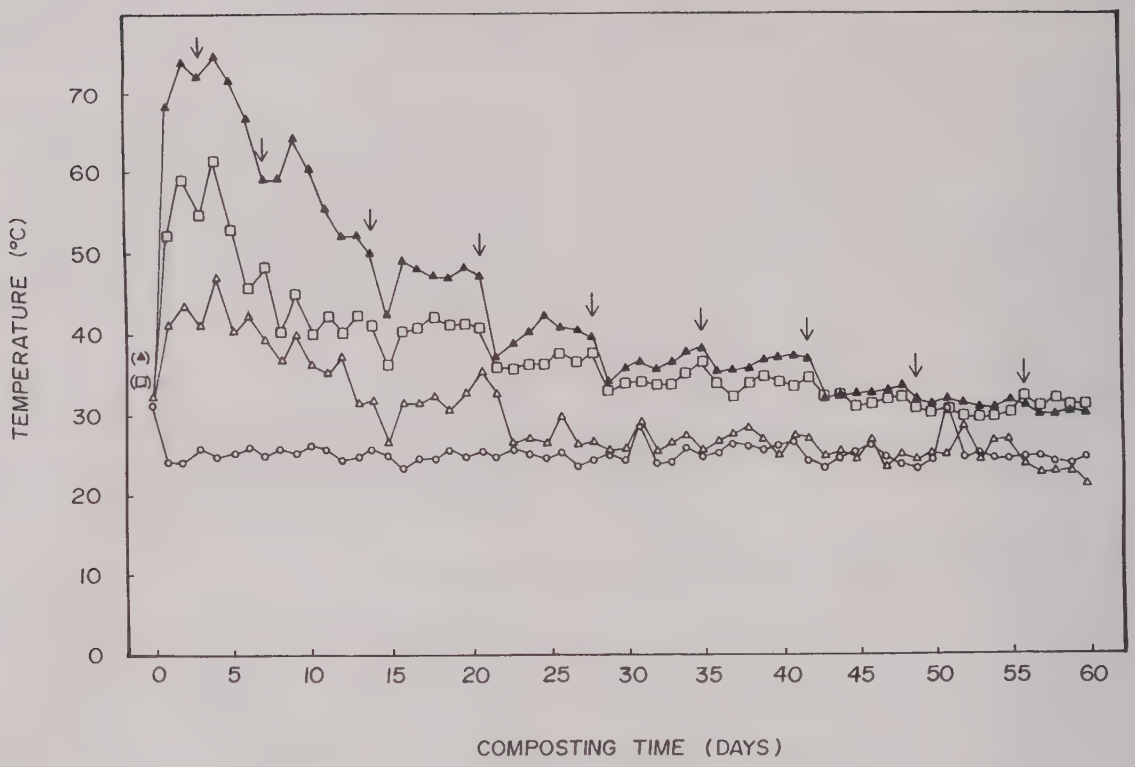

Fig. 3. Temperature–time plot of the top △, middle ▲ and bottom □ layers of Heap 2.

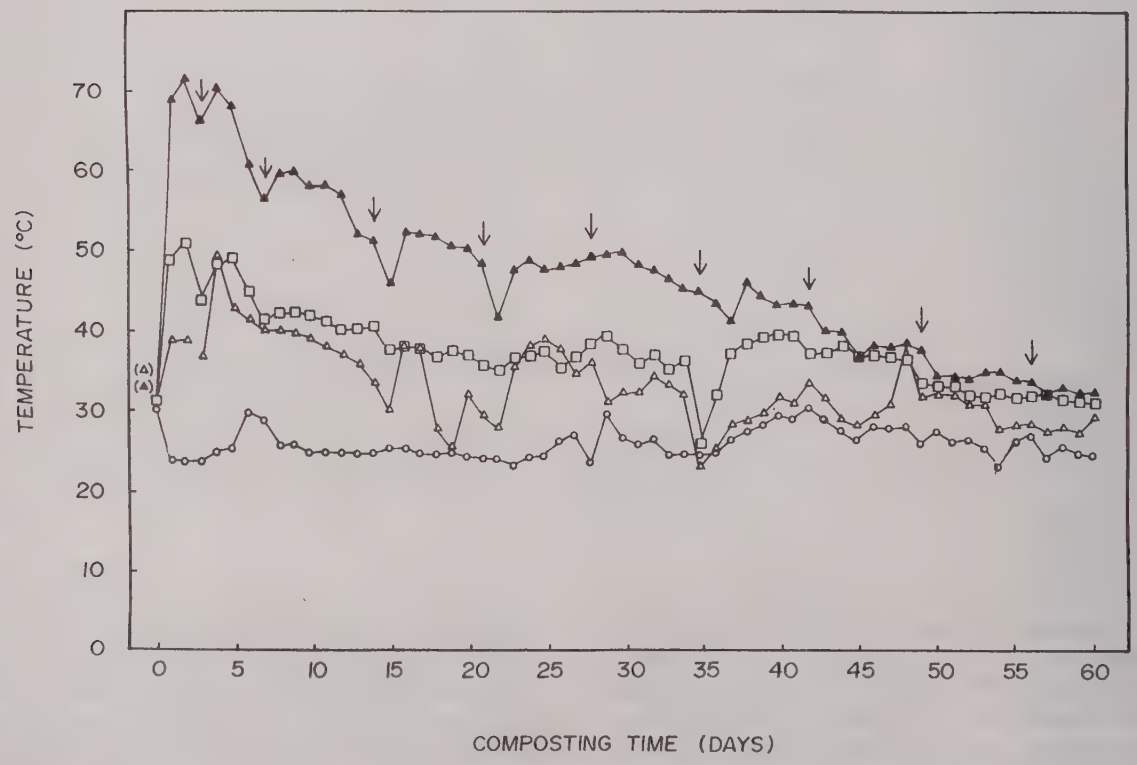

Fig. 4. Temperature–time plot of the top △, middle ▲ and bottom □ layers of Heap 3.

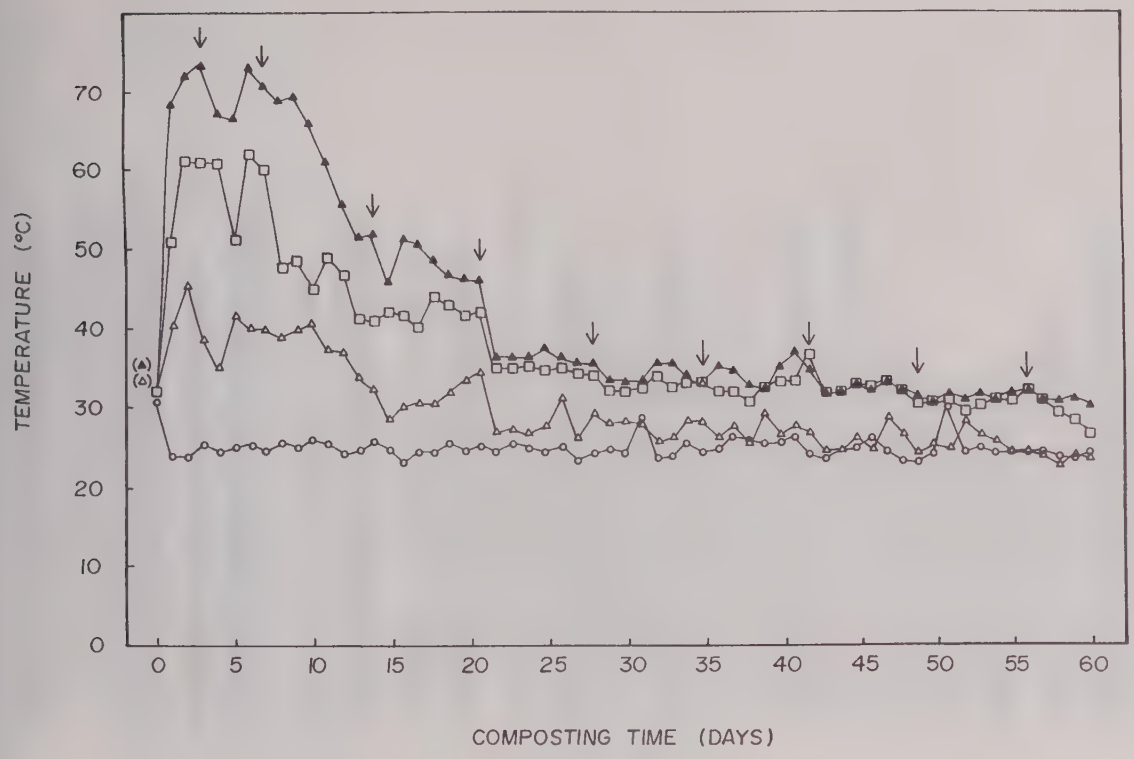

Fig. 5. Temperature–time plot of the top △, middle ▲ and bottom □ layers of Heap 4.

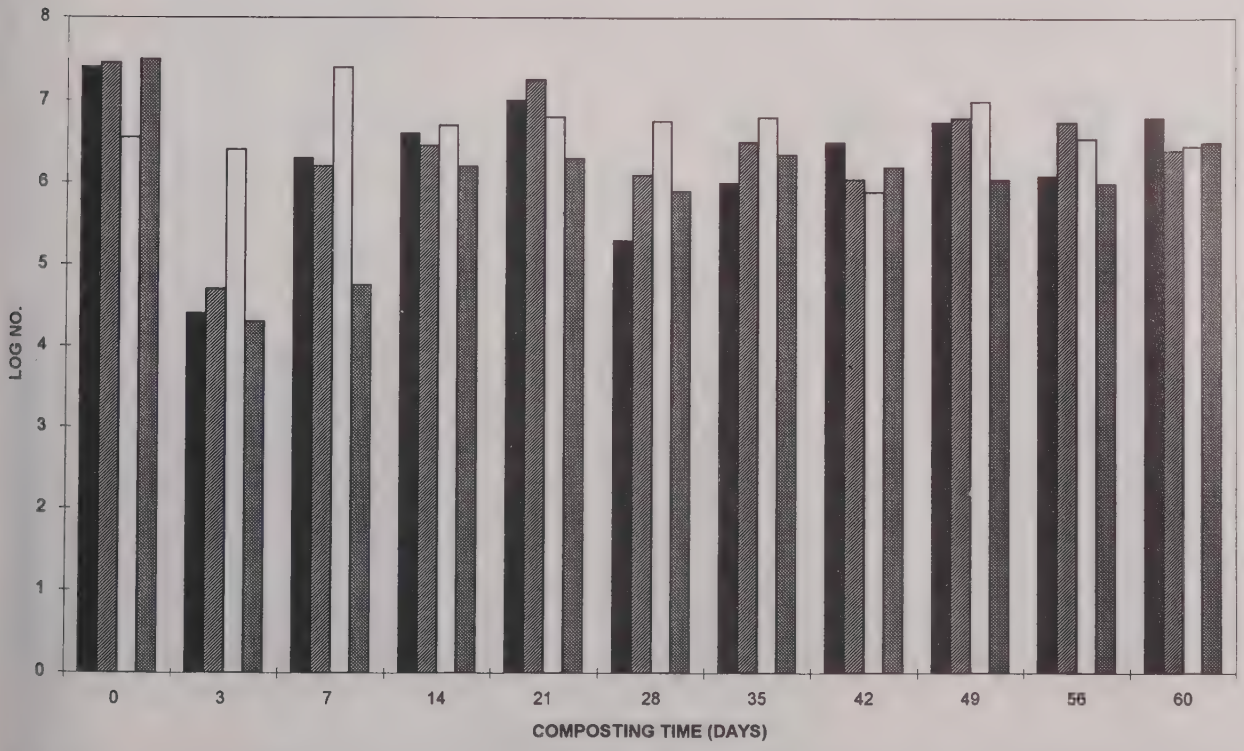

Fig. 6. Log numbers of mesophilic bacteria during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

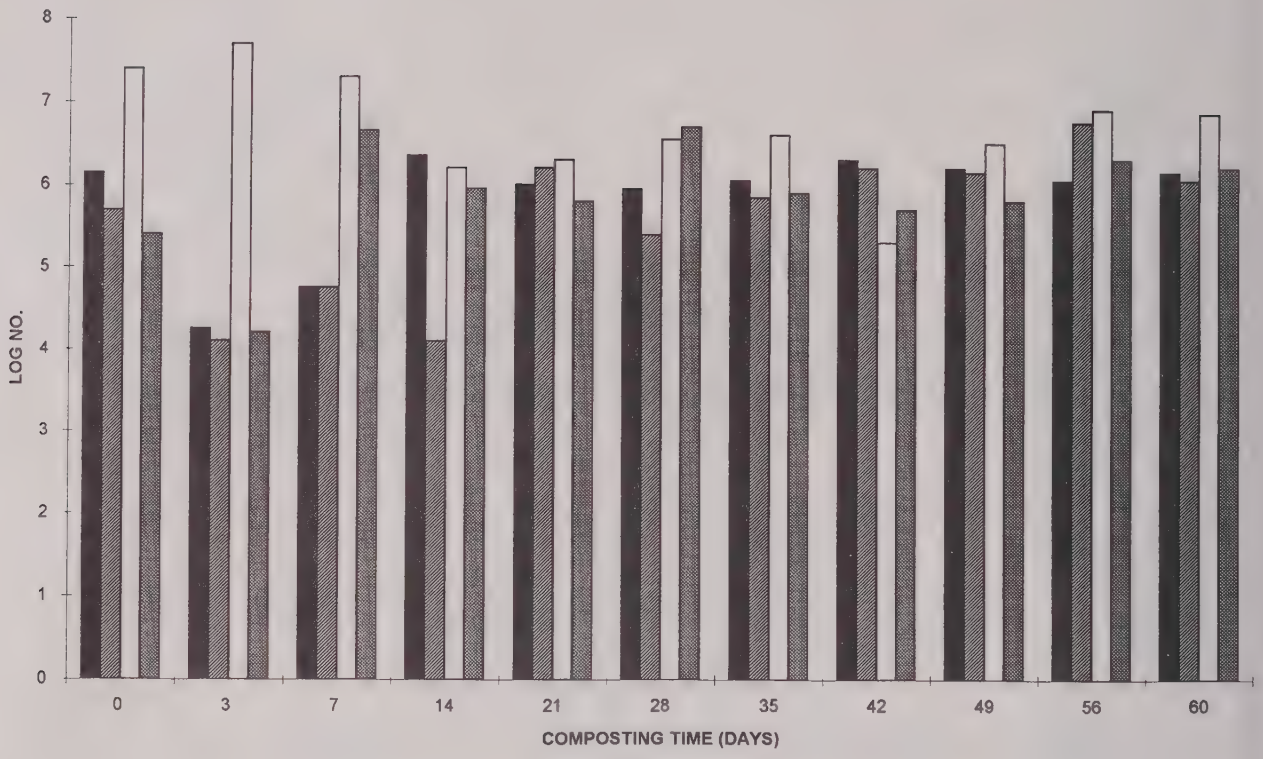

Fig. 7. Log numbers of thermophilic bacteria during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

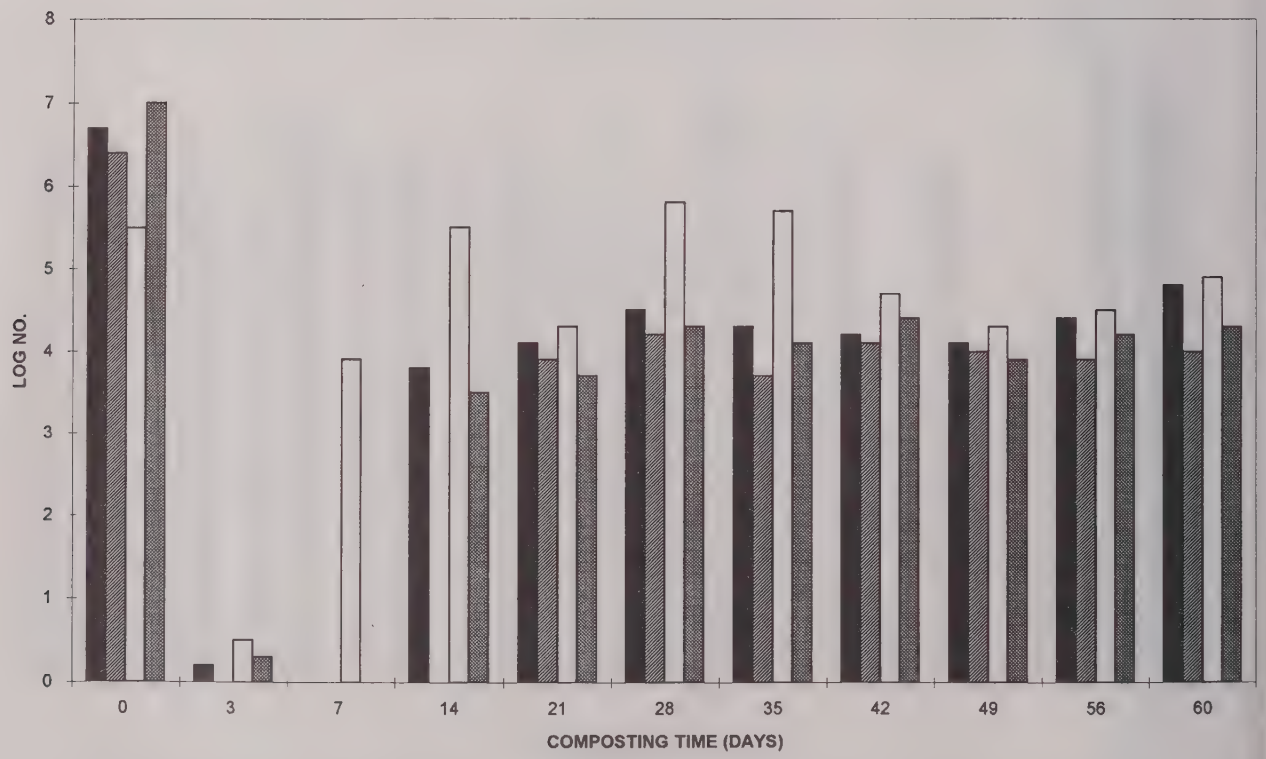

Fig. 8. Log numbers of mesophilic fungi during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

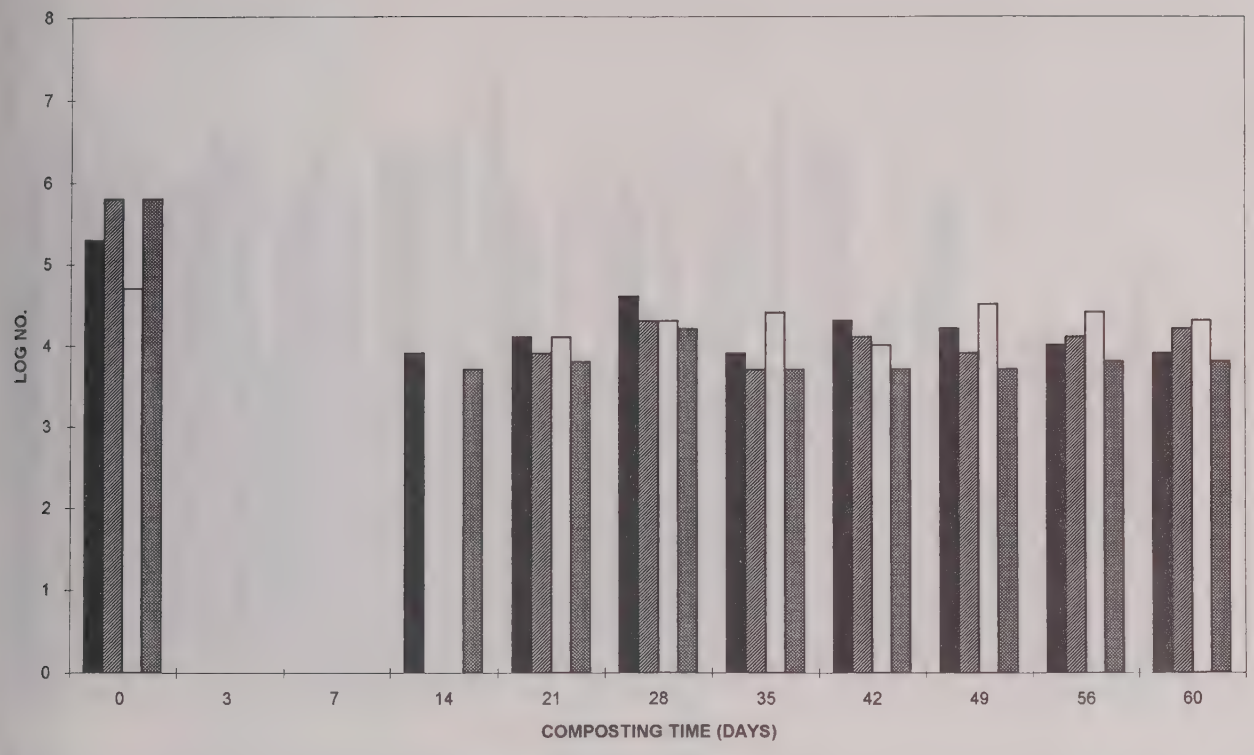

Fig. 9. Log numbers of thermophilic fungi during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

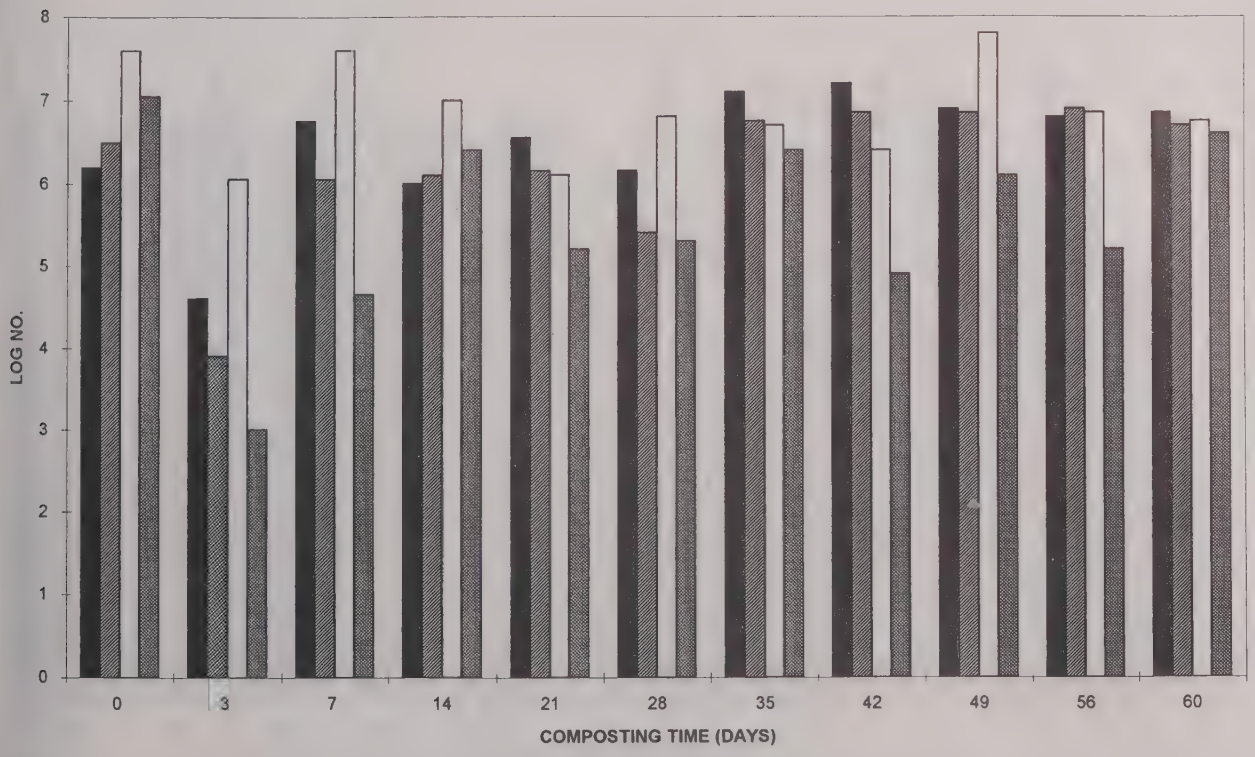

Fig. 10. Log numbers of mesophilic actinomycetes during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

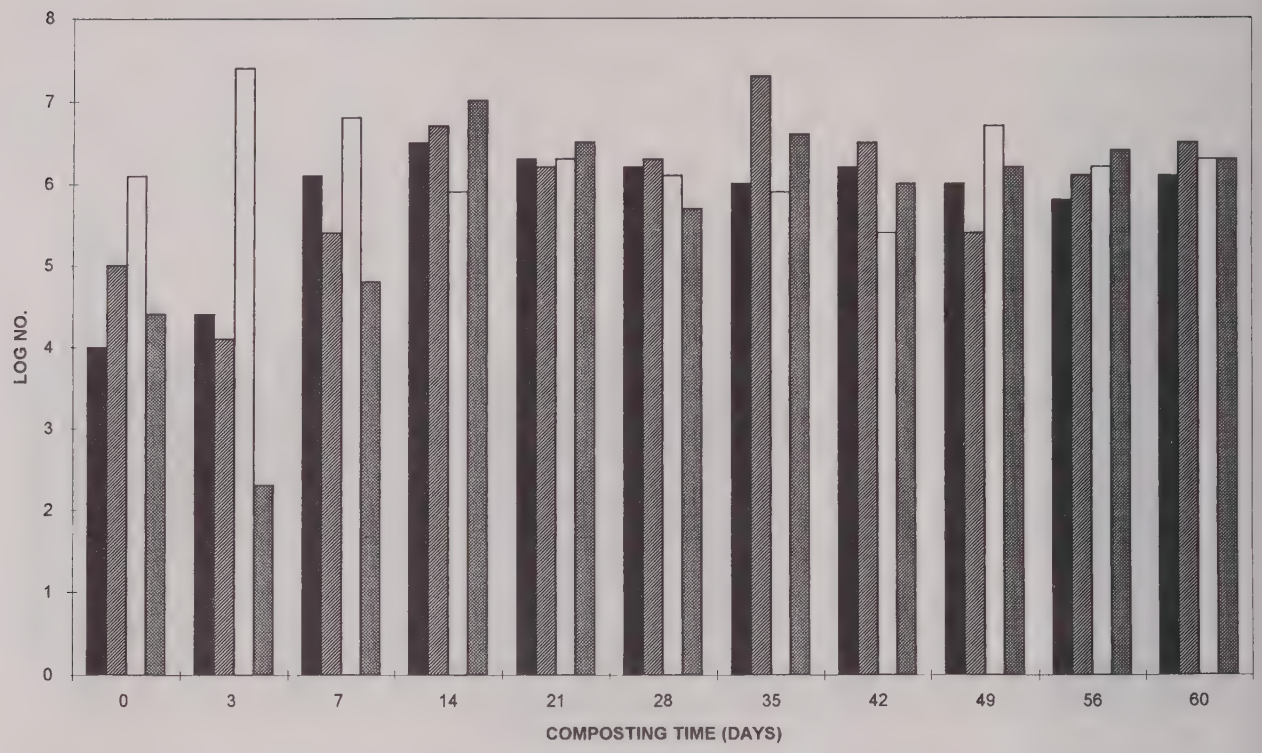

Fig. 11. Log numbers of thermophilic actinomycetes during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

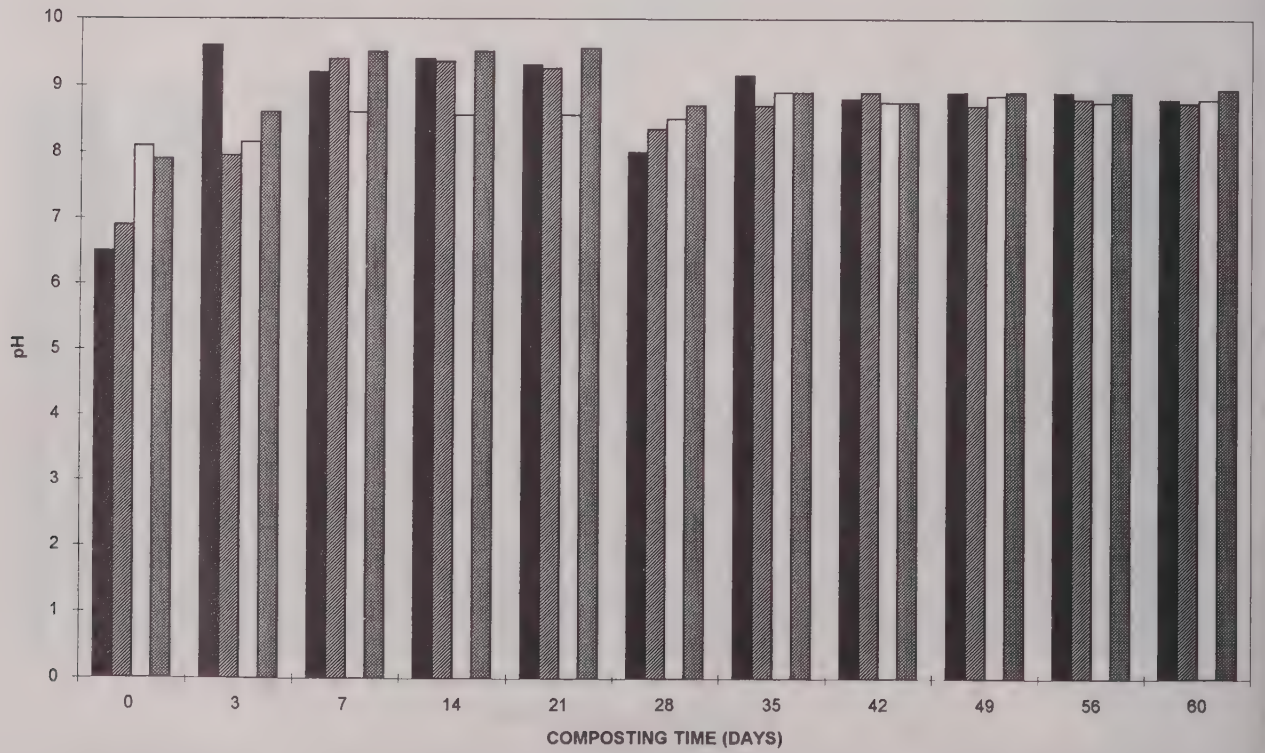

Fig. 12. The pH of the substrate during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

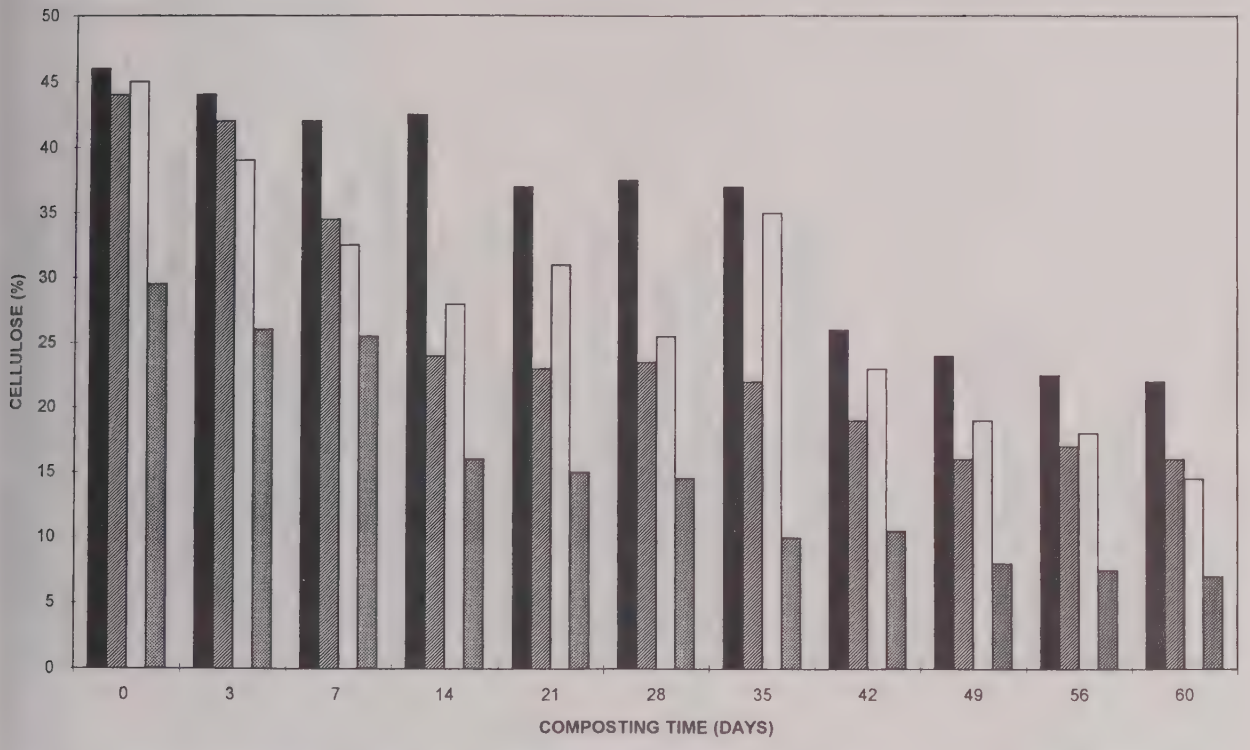

Fig. 13. Cellulose concentration during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

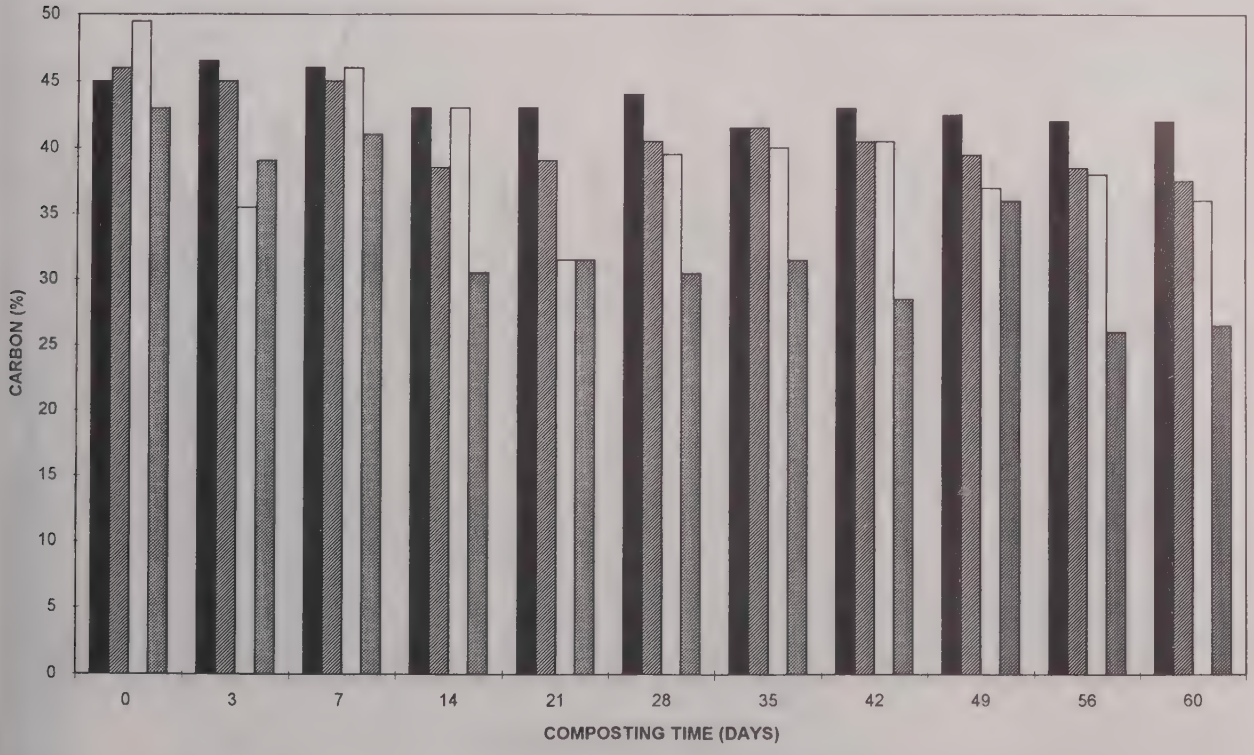

Fig. 14. Carbon concentration during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

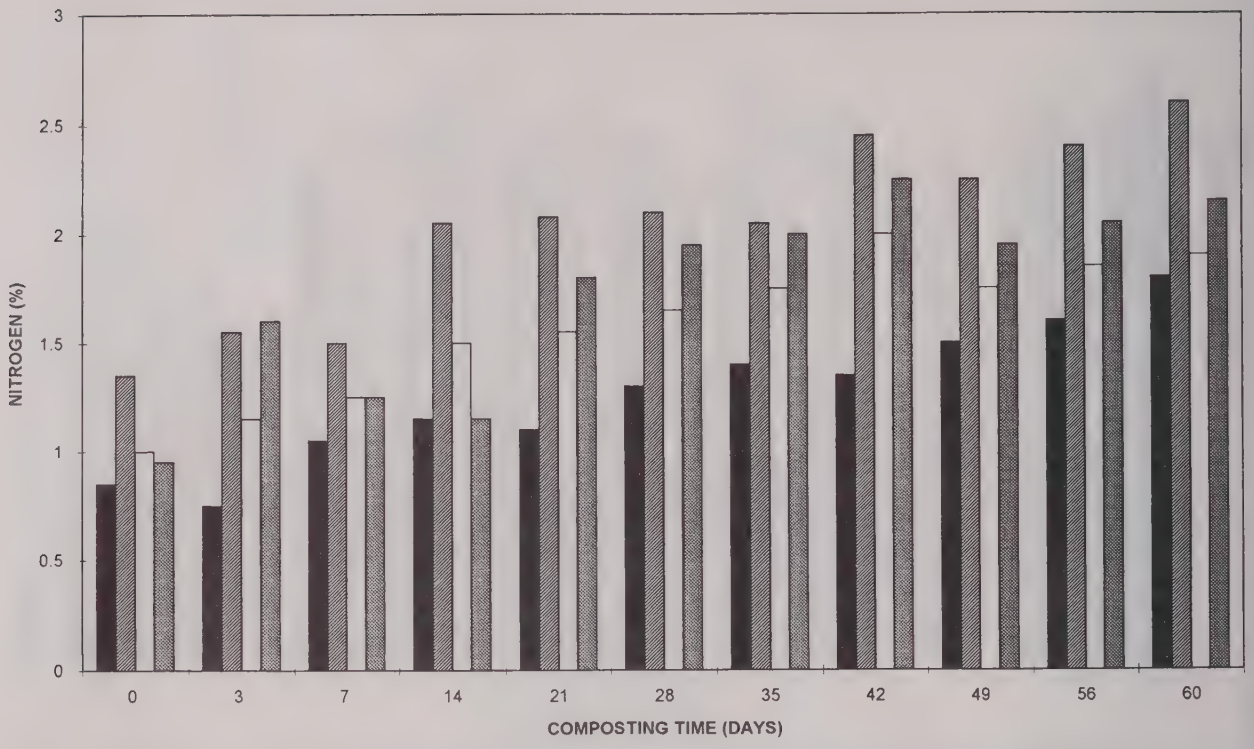

Fig. 15. Nitrogen concentration during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

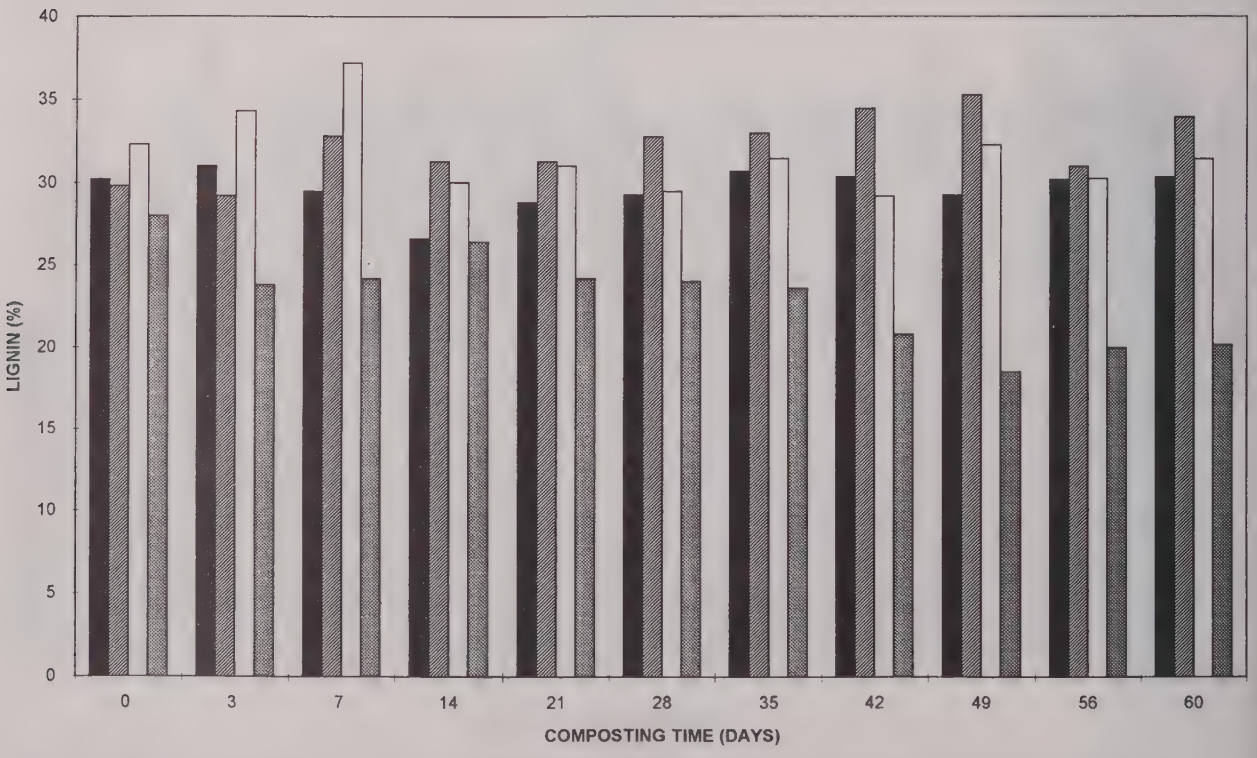

Fig. 16. Lignin concentration during the composting process. ■, Heap 1; ▨, Heap 2; □, Heap 3; ▩, Heap 4.

Table 1. ANOVA of C:N ratios, cellulose, lignin and pH with respect to heap and composting time

Source of variation	C:N ratio				Cellulose conc.				Lignin				pH			
	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
Co-variants (day)	2802	1	96	0·00	2928	1	273	0·00	4	1	1	0·31	1	1	2	0·09
Main effects (heap)	1757	3	20	0·00	2010	3	61	0·00	569	3	46	0·00	0·83	3	0·73	0·53
Residual	1102	38			425	39			157	39			14	39		
Total (corr.)	5662	42			5418	43			731	43			16	43		

I — Sum of squares.
II — d.f.
III — F-ratio.
IV — Sig. level.

between 5·4 and 8·2, and an average of 9·5 was achieved at the end of the process (Fig. 12). Nyns (1986) observed losses in ammonia in compost heaps which had low carbon:nitrogen ratios. However, nitrogen is generally stabilized in the process by being transformed into microbial protein.

Microbial decomposition of plant material resulted in an overall loss of cellulose and carbon (Figs 13 and 14) and an increase in nitrogen content (Fig. 15). These changes, which resulted from microbial activity on the cellulosic substrate and nitrogen from the dungs, increased microbial protein and humic substances. Although lignolytic microorganisms occur in compost heaps (Fergus, 1964), the breakdown of lignin was not noted (Fig. 16), concurring with an earlier report on palm press fibre (Thambirajah & Kuthubutheen, 1989).

Statistical analyses showed highly significant differences for changes in C:N ratio and cellulose in the four heaps during the composting process (Table 1). No significant difference was observed for changes in lignin and pH.

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AN ENERGY BUDGET AND EFFICIENCY OF SEWAGE-FED FISH PONDS

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Abstract

An energy computation of a serially-connected, sewage-fed fish pond system was made. The annual solar radiation was 4456·2 MJ/m^2. The synthetic efficiency of solar radiation to different fish ponds varied from 0·46 to 1·24%. The total energy input and phytoplankton production in ponds ranged from 11328 to 29082·2 MJ/y and 8·89 to 23·60 kg/m^2/y, respectively. The gross conversion efficiencies of total, photosynthetic, biological and biological-industrial energy varied from 0·58 to 3·3%, 1·5 to 6%, 0·95 to 11·1% and 0·92 to 10%, respectively, in different fish ponds. The gross energy consumption for the production of fish varied from 116·9 to 732·5, 68·9 to 275·8, 34·9 to 443·7 and 38·9 to 456·8 MJ/kg fish for total, solar, biological and biological-industrial energy, respectively, in different ponds. The selection of fish species and maintenance of optimum physico-chemical and biological parameters are suggested to improve the energy transformation in sewage-fed fish ponds.

Key words: Sewage, fish culture, solar radiation, phytoplankton, energy conversion, energy consumption.

INTRODUCTION

The increase in the human population has increased the volume of domestic sewage. The wastewater from human settlements, rich in nutrients, is now regarded as a good source of fertilizer. Sewage-fed fish culture has been practised in India for centuries (Bose, 1944; Chatterjee *et al*., 1967; Ghosh *et al*., 1974; Govindan, 1989; Raj, 1990; Balasubramanian *et al*., 1992). Sewage-fed aquaculture not only promotes fish production but offers a successful means of sewage disposal.

In intensive aquaculture systems, both feed and fertilizer are considered as major external inputs. In line with the above fact, Blaxter (1975), Edwardson (1976) and Pitcher and Phil (1977) computed only biological and industrial energy and did not consider the solar energy for energy budgets in trout fish-farms. In organic-waste-fed fish-culture systems, apart from biological and industrial energy, solar energy plays an important role in the production of live feed organisms and active decomposition of organic compounds. Thus it is all the more necessary to include the solar energy as one of the inputs in an energy budget, especially in tropical situations. Energy structure and efficiency studies were carried out in certain conventional, integrated, fish farms (Li, 1984; 1987). However, an energy-budget study of a sewage-fed fish-culture system cannot be found in the literature. Hence, the present study was made to present an energy budget for a sewage-fed fish-culture system. The general running and results from the system have been described in previous papers (Balasubramanian *et al*., 1992; 1995).

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METHODS

Experimental site

Sewage from the university campus was fed from a collection tank to three stabilization ponds and then to six fish ponds in cascade. The ponds were each of 200 m^2 area and the total retention time was 16·1 days. The experiments were carried out from October 1992 to July 1993. Complete details are given by Balasubramanian *et al*., 1995.

Fish stocking details

Fish were stocked in all the six fish ponds after a one month stabilization of the ponds. The number of various fish species stocked per pond, initial weight and gross yield obtained in each species in different fish ponds are shown in Table 1. In the first fish pond (P_1), BOD and diurnal oxygen fluctuations were very high, hence it was stocked with the air-breathing murrel, *Channa striatus*. In ponds 2 and 3 (P_2 and P_3), the omnivorous tilapia, *Oreochromis mossambicus* (male — visual identification of genitals) was stocked as this can tide over high physico-chemical changes in ponds. In ponds 4, 5 and 6 (P_4, P_5 and P_6), the oxygen fluctuations were

Table 1. Stocking and harvesting details of fish in sewage-fed fish ponds of 200 m^2 each

Fish species stocked	No. of fish/ pond	Average initial weight (g)	Gross yield (kg)					
			1	2	3	4	5	6
Channa striatus	100	4·4	39·8					
Oreochromis mossambicus (mono sex)	400	5·2		31·5	48·9			
Hypophthalmichthys molitrix	40	1·4				8·1	21·9	29·1
Ctenopharyngodon idella	10	3·0				3·1	4·1	5·6
Cyprinus carpio var. *communis*	40	0·8				14·0	19·7	21·5
Catla catla	30	1·1				7·7	14·3	22·2
Labeo rohita	40	1·0				13·8	16·7	28·4
Cirrhinus mrigala	40	7·7				13·3	16·2	19·4
Total production (kg)			39·8	31·5	48·9	60·0	92·9	126·2

smaller. Hence, these ponds were stocked with the Indian major carp and Chinese carp. No supplementary feed was given to the fish.

Input and output estimation

All inputs and outputs involved in the system were converted into energy equivalent (Joules). Various energy inputs involved in the system were classified into three distinct groups for the purpose of convenience (Li, 1987):

Group	*Energy involved*
Photosynthetic	Solar
Biological	Sewage organics
Industrial	Fingerlings, labour, electricity and maintenance

Calorific values of sewage collected from the sump and inlet regions of different fish ponds were determined fortnightly by the method of Johnson (1977). Known amounts of water samples were taken from the ponds individually in silica crucibles and dried overnight at 60°C on a water bath and weighed to obtain the dry weight. Known amounts of the dry residues were burned in a micro-bomb calorimeter to determine the energy value. Thus the energy value of the total sewage loaded in each pond over a period of 300 days was calculated.

The primary productivity study in all ponds was carried out fortnightly to find out the input of solar energy. The light and dark bottle method was used to study the primary productivity (APHA, 1985). Analyses were made four times a day from 8 to 9 a.m., from 11 to 12 noon, from 2 to 3 p.m. and from 5 to 6 p.m. and the average primary production per hour was calculated. This value was multiplied by the total average solar insolation of the day to arrive at a daily total primary production. Productivity studies were carried out on all levels of the standing water: surface, column and bottom, and all the three zones were averaged for the purpose of calculating the total productivity. The productivity of the water was expressed in terms of oxygen produced per 1 m^2 of surface for each hour. From the above, the yearly (300 days) values were worked out. The oxygen value was converted into energy values (J) by multiplying by an energy equivalent (mg $O_2 \times 3{\cdot}38 \times 4{\cdot}18$). The oxygen value was also used to determine the wet weight of phytoplankton (Wang & Shen, 1981). Accordingly, 1 g O_2 evolves from 6·1 g wet weight of phytoplankton and 444·7 g wet weight of phytoplankton yields 1 MJ of energy.

Energy values of fish fingerlings were assessed using a micro-bomb calorimeter. After 300 days of culture all the ponds were drained and fish were collected. The representative fish in each species were brought to the laboratory, chopped to pieces, dried at 103°C, powdered and the energy values were determined. The total energy yield through the fish for each pond was calculated by multiplying the energy value of different fish species by their corresponding production.

Estimation of ecological efficiency

The gross total output was calculated from the total fish harvested. The net total output was calculated by subtracting the energy of fingerlings stocked from the gross energy yield. The total input was calculated by adding the energy values of photosynthetic, biological and industrial energy. Methods followed by Li (1987) were used for the purpose of calculating the energy conversion efficiencies and energy consumption pattern.

Formulae used to calculate the energy conversion efficiency (Li, 1987):

$$\text{Gross total energy conversion efficiency (\%)} = \frac{\text{Gross total output (MJ)}}{\text{Total input (MJ)}} \times 100$$

Net total energy conversion efficiency (%)

$$= \frac{\text{Net total output (MJ)}}{\text{Total input (MJ)}} \times 100$$

Gross biological energy conversion efficiency (%)

$$= \frac{\text{Gross total output (MJ)}}{\text{Biological input (MJ)}} \times 100$$

Net biological energy conversion efficiency (%)

$$= \frac{\text{Net total output (MJ)}}{\text{Biological input (MJ)}} \times 100$$

Gross biological–industrial energy conversion efficiency (%)

$$= \frac{\text{Gross total output (MJ)}}{\text{Biological} + \text{industrial input (MJ)}} \times 100$$

Net biological–industrial energy conversion efficiency (%)

$$= \frac{\text{Net total output (MJ)}}{\text{Biological} + \text{industrial input (MJ)}} \times 100$$

Gross photosynthetic energy conversion efficiency (%)

$$= \frac{\text{Gross total output (MJ)}}{\text{Photosynthetic energy input (MJ)}} \times 100$$

Net photosynthetic energy conversion efficiency (%)

$$= \frac{\text{Net total output (MJ)}}{\text{Photosynthetic energy input (MJ)}} \times 100$$

Synthetic efficiency for solar radiation (%)

$$= \frac{\text{Primary production (MJ/m}^2\text{/y)}}{\text{Solar radiation (MJ/m}^2\text{/y)}} \times 100$$

Formulae used to calculate the energy consumption estimate (Li, 1987):

Total energy consumption per unit of gross yield (MJ/kg fish)

$$= \frac{\text{Total input (MJ)}}{\text{Gross output (kg)}}$$

Total energy consumption per unit of net yield (MJ/kg fish)

$$= \frac{\text{Total input (MJ)}}{\text{Net output (kg)}}$$

Biological energy consumption per unit of gross yield (MJ/kg fish)

$$= \frac{\text{Biological input (MJ)}}{\text{Gross output (kg)}}$$

Biological energy consumption per unit of net yield (MJ/kg fish)

$$= \frac{\text{Biological input (MJ)}}{\text{Net output (kg)}}$$

Biological–industrial energy consumption per unit of gross yield (MJ/kg fish)

$$= \frac{\text{Biological} + \text{industrial input (MJ)}}{\text{Gross output (kg)}}$$

Biological–industrial energy consumption per unit of net yield (MJ/kg fish)

$$= \frac{\text{Biological} + \text{industrial input (MJ)}}{\text{Net output (kg)}}$$

Solar energy consumption per unit of gross yield (MJ/kg fish)

$$= \frac{\text{Solar energy input (MJ)}}{\text{Gross output (kg)}}$$

Solar energy consumption per unit of net yield (MJ/kg fish)

$$= \frac{\text{Solar energy input (MJ)}}{\text{Net output (kg)}}$$

RESULTS AND DISCUSSION

The main aim of a sewage-fed fish culture system is to utilize the available nutrients in the sewage to produce table-size fish and to simultaneously allow recycling of the sewage to the maximum extent. In the present study the major input items were sewage, photosynthetic energy, fish fingerlings, labour, electricity and maintenance. The output was the total fish yield.

The monthly solar radiation (MJ/m^2/month) and its percentage distribution is shown in Table 2. The

Table 2. Monthly solar radiation and percentage distribution during the experimental period

Month	Solar radiation (MJ/m^2/month)	Monthly distribution of solar radiation (%)
1992		
Oct.	268·3	6·0
Nov.	337·8	7·6
Dec.	339·6	7·6
1993		
Jan.	506·3	11·4
Feb.	543·0	12·2
Mar.	556·1	12·5
Apr.	564·2	12·6
May	422·5	9·5
June	498·5	11·2
July	419·9	9·4
Annual radiation (300 days)	4456·2	100

solar radiation was highest during April 1993 and lowest during October 1992. The synthetic efficiency for solar radiation in different fish ponds varied from 0·46 to 1·24% (Table 3). There are no parallel data in the literature regarding the synthetic efficiency of energy in sewage-fed ponds. Li (1989) reported that 1·15% solar synthetic efficiency was observed in an integrated fish-farm in China. For natural waters, such as lakes, the corresponding values are only 0·1–0·5% (Lindeman, 1942; Odum, 1971; Wang & Shen, 1981). It would seem that the synthetic energy obtained in the present work was higher than the values reported.

Table 3. Synthetic efficiency for solar radiation in different ponds (%)

Pond 1	Pond 2	Pond 3	Pond 4	Pond 5	Pond 6
1·24	0·79	0·46	0·77	0·71	1·12

Table 4. Sewage energy (biological energy) input in sewage-fed fish ponds (200 m^2) per year

Pond	Quantity of sewage loaded in serially-connected fish ponds (m^3/y)	Energy value of sewage (J/l)	Energy input through sewage in each fish pond (MJ/y)
1	20610·0	3620·7	17615·1
2	19789·8	2880·6	10185·9
3	19120·9	2448·7	8592·8
4	18326·2	2086·3	4583·2
5	17632·1	1908·5	4522·7
6	16937·9	1719·7	4403·6
Outflow from P6	16260·8	1520·5	—

Table 5. Phytoplankton biomass production and primary production in six sewage-fed fish ponds

Pond	Phytoplankton biomass production (kg/m^2/y)	Primary production (MJ/m^2/y)
1	23·60	55·30
2	15·26	35·36
3	8·89	20·60
4	14·73	34·13
5	13·99	31·77
6	21·90	49·74

Table 4 shows the sewage energy (biological energy) input into the sewage-fed ponds per year. The energy input through sewage gradually reduced from ponds 1 to 6. This was mainly due to the gradual reduction of organic matter and nutrients in the serially-connected ponds.

The production of phytoplankton biomass and consequent primary production in the fish ponds are shown in Table 5. The phytoplankton production level varied in different ponds. It was high in pond 1 and low in pond 3. As indicated earlier, phytoplankton biomass was calculated from the dissolved oxygen production. The high phytoplankton biomass production in pond 1 was mainly due to nutrient enrichment in that water. Due to zooplankton dominance the phytoplankton production was low in pond 3. The calculated value for phytoplankton biomass production in different ponds was much higher than that of natural lakes. Wang and Liang (1981) estimated a phytoplankton production of 6·12 kg/m^2/y in their fish farm in Donghu lake in China. The fish farm in Honghu lake in China (35·5 ha) had a phytoplankton biomass production of 4·93 kg/m^2/y (Cheng, 1983). Olah *et al.* (1986) calculated that the phytoplankton biomass production in six sewage-fed fish ponds in Hungary varied from 0·98 to 3·12 kg/m^2/300 days.

An energy budget of the different ponds is shown in Table 6. The total energy input into the ponds varied from 11328 to 29082 MJ/y. This total energy input variation was mainly due to variation in photosynthetic and sewage-energy inputs. The fish yield in ponds in terms of energy output varied from 109·3 to 490 MJ/y. The fish yield was mainly dependent on physico-chemical and biological parameters existing in the ponds.

The energy conversion efficiencies (photosynthetic, biological, biological–industrial and total) of the different fish ponds also varied (Table 7). The gross conversions of photosynthetic, biological, biological–industrial and total energy were low in pond 1, but the gross efficiencies of biological, biological–industrial and total energy values were higher in pond 6 than other ponds. The gross photosynthetic energy conversion efficiency was higher in pond 5

Table 6. Energy budget of the sewage-fed fish-culture system

Pond	Input (MJ/y)						Output (MJ/y)
	Photosynthetic	Sewage	Fingerlings	Labour	Electricity and maintenance	Total energy input	Total fish yield
1	10949·40	17615·11	3·68	220·0	294·0	29082·20	167·07
2	7000·88	10185·91	7·28	220·0	294·0	17708·10	109·29
3	4077·81	8592·81	7·28	220·0	294·0	13191·90	169·93
4	6758·14	4583·20	0·96	220·0	294·0	11856·30	227·57
5	6290·46	4522·68	0·96	220·0	294·0	11328·00	374·46
6	9849·11	4403·58	0·96	220·0	294·0	14767·70	490·00

Table 7. Energy conversion efficiency in different fish ponds (%)

Pond	Photosynthetic energy		Biological energy		Biological–industrial energy		Total energy	
	Gross	Net	Gross	Net	Gross	Net	Gross	Net
1	1·526	1·525	0·948	0·949	0·921	0·922	0·575	0·574
2	1·561	1·563	1·072	1·073	1·021	1·021	0·620	0·617
3	4·167	4·175	1·976	1·977	1·864	1·865	1·290	1·288
4	3·367	3·368	4·964	4·944	4·464	4·465	1·920	1·919
5	5·953	5·954	8·504	8·258	7·433	7·434	3·310	3·305
6	4·975	4·975	11·127	11·105	9·964	9·964	3·321	3·318

Table 8. Energy consumption pattern in different fish ponds (MJ/kg fish)

Pond	Solar energy		Biological energy		Biological–industrial energy		Total energy	
	Gross	Net	Gross	Net	Gross	Net	Gross	Net
1	275·80	304·00	443·70	489·06	456·75	503·48	732·50	807·44
2	222·25	289·10	323·36	420·63	339·91	442·15	562·16	731·26
3	83·39	97·99	175·72	206·48	168·38	219·01	269·77	316·99
4	112·64	114·50	76·39	77·65	84·97	86·37	197·61	197·58
5	68·88	68·80	48·95	49·47	54·52	55·10	122·60	123·90
6	77·98	78·59	34·87	35·13	38·94	39·25	116·93	117·84

than in the other ponds. The gross photosynthetic energy conversion efficiency of six sewage-fed fish ponds studied in Hungary varied from 1·6 to 5·2% (Olah *et al.*, 1986). In Nanhui integrated fish-farm, China, the biological energy conversion efficiency was 10·5% (Li, 1987). Such a high level of conversion was achieved only in the sixth fish pond of the present system. Pimentel (1980) reported 10·4% as the biological–energy conversion efficiency for a polyculture system (silver carp, common carp, tilapia and mullet) in Israel.

Table 8 shows the energy consumption pattern (MJ/kg fish) in different ponds. All types of energy consumption were highest in pond 1 and lowest in pond 6. In pond 6 the gross biological energy consumption was 34·9 MJ/kg fish. The biological energy consumption in Nanhui integrated fish-farm was 46·7 MJ/kg fish. Pitcher and Phil (1977) estimated the biological energy consumption as 55·8 MJ/kg fish in a rainbow-trout farm with running water. The energy consumption of a catfish farm in Thailand was 56 MJ/kg fish (Edwardson, 1976). In comparison it is clear that the energy consumption in the sixth fish pond in the present system was the lowest (34·9 MJ/kg fish).

In the present system the ecological efficiency (energy conversion and energy consumption) was better in the sixth fish pond than in other ponds. The physico-chemical and biological parameters (diurnal oxygen fluctuation, plankton species and productivity) of the sixth fish pond were nearer optimum for fish growth than in the other ponds. The selection of fish species in a polyculture system, and the optimum physico-chemical and biological parameters in a sewage-fed pond, will improve the input–output structure and ecological efficiency. Sewage, being a low energy-input system, was observed to be supreme in energy conversion when it was recycled through fish culture, especially when the fish were not fed with supplementary feeds.

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TERTIARY TREATMENT OF CHEESE FACTORY ANAEROBIC EFFLUENT WITH *PHORMIDIUM BOHNERI* AND *MICRACTINIUM PUSILLUM*

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Abstract

The growth and nutrient removal capacity of the cyanobacterium Phormidium bohneri *and of the endogenous microalga* Micractinium pusillum *were investigated for the biotreatment of a cheese factory anaerobic effluent.* Phormidium bohneri *demonstrated higher growth rate* ($k = 0{\cdot}62\ d^{-1}$) *and biomass yield (329 mg dry mass* l^{-1}*) than that of* M. pusillum *(0·35* d^{-1} *and 137 mg dry mass* l^{-1}*) over four days. In the presence of this cyanobacterium or this microalga, ammonia was completely removed after four days, although the kinetics of removal were different for both species. Rates of removal of phosphorus in the presence of* P. bohneri *and* M. pusillum *were 2·9 and 2·5 mg* $P\text{-}PO_4^{3-}.l^{-1}.d^{-1}$*, respectively. These results were interpreted as a function of different factors, such as the increase in pH and aeration time.*

Key words: Ammonia, biological treatment, cheese factory effluent, *Micractinium pusillum*, *Phormidium bohneri*, phosphorus.

INTRODUCTION

Microalgal cultures offer interesting alternatives for wastewater treatment as tertiary biotreatments to remove inorganic nutrients, such as nitrogen and phosphorus, while producing potentially valuable biomass (de la Noüe *et al.*, 1992). Effluents from secondary domestic wastewater treatment plants and from anaerobically-digested animal wastes are the most common types of wastes used for the growth of microalgae and cyanobacteria. These effluents are well suited for the growth of algae because they contain high amounts of inorganic nutrients and relatively low amounts of organic compounds. In addition, recent publications indicate that microalgae and cyanobacteria can also be grown, given appropriate dilution, in other types of effluents, such as *Spirulina* on starch wastewater (Tanticharoen *et al.*, 1994), *Anacystis* on dairy and sugar cane wastewaters (Thangaraj & Kulandaivelu, 1994) and *Chlorella* on palm-oil mill effluent (Aziz & Ng, 1992). Because *Phormidium bohneri* shows potential for removing nitrogen and phosphorus from domestic wastewaters (Proulx *et al.*, 1994), we decided to investigate the potential utilization of this cyanobacterium for the tertiary treatment of anaerobically-treated cheese factory effluents. Although anaerobic treatment reduces the organic load of this type of waste, the effluent is still rich in inorganic nitrogen and phosphorus. Growth and rates of removal of ammonium and phosphorus by *P. bohneri* were compared with those of the unicellular colonial green alga *Micractinium pusillum*, a species endogenous in ponds receiving effluent from the cheese factory.

METHODS

Organisms

Phormidium bohneri (Schmidle) was grown in monospecific but not axenic stock cultures at 25°C in liquid mineral medium with KNO_3 as the nitrogen source (Dauta, 1982). The biomass was concentrated by filtration, washed with distilled water, homogenized with a tissuemizer (Tekmar TP 18/10S1), left in darkness overnight at 4°C, and washed once in mineral medium deprived of nitrogen and phosphorus before the beginning of the experiments. *Micractinium pusillum* was isolated from aerated ponds in effluent from the AGROPUR cheese factory (Notre-Dame-du-Bon-Conseil, Québec, Canada). It was grown in batch cultures and fed with cheese factory effluent diluted at 5% (v/v) until its biomass concentration reached 660 mg dry mass l^{-1} (mg d.m. l^{-1}). Because it was not possible to wash the cells without damaging them, inoculation of the replicates was done with cells grown for two days in the absence of an added source of nitrogen and phosphorus, in order to begin the experiment with a

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medium containing a low amount of residual inorganic nutrients.

Experimental system

Wastes from the AGROPUR cheese factory were firstly treated by anaerobic digestion in a multiplate reactor, followed by aerobic digestion in four sequential aeration ponds before decantation in two sedimentation ponds and discharge of the effluent in the Nicolet river. The composition of the effluent was: N-NH_3, 125 mg l^{-1}; P-PO_4^{3-}, 80 mg l^{-1}; N-NO_2^--NO_3^-, less than 1 mg l^{-1}; total solids (TS), 3600 mg l^{-1}; total suspended solids (TSS), 900 mg l^{-1} and total COD, 1500 mg l^{-1}. Anaerobically-treated wastewater from the cheese factory treatment plant was brought back to the laboratory and stored a few days in covered plastic containers, in the dark at 4°C, in order to lower the suspended solids from an initial value of 900 mg to about 180 mg dry mass l^{-1}. During the experiment, *Micractinium pusillum* and *Phormidium bohneri* were grown in batch cultures at 21±1°C with a light intensity of 80 μmol m^{-2} s^{-1} and a 15:9 h light:dark cycle. Cultures were done in plastic bottles ($n = 4$) with an initial volume of 1·8 l, an aeration flow rate of 1 l^{-1} min^{-1} and an inoculum of 111 mg d.m. l^{-1}. Initial concentrations of 25 mg N-NH_3 l^{-1}, 16 mg P-PO_4^{3-} l^{-1} and 36 mg l^{-1} TSS were obtained from a 20% dilution of the settled effluent.

Biomass density determination

The algal biomass was filtered through tared Whatman glass microfilters (934-AH), dried at 95°C for 24 h, and then transferred to a dessicator before weighing. However, in the first hours following the transfer of *P. bohneri* in cheese factory anaerobic effluent, we observed a very rapid increase in dry mass. This increase was proportional to the amount of *P. bohneri* biomass put in the presence of the effluent (data not shown). Upon microscopic examination of the flocs formed, we concluded that this increase in dry mass was due to an initial adhesion of the organic matter present in the cheese factory anaerobic effluent on the algal flocs and that this phenomenon became negligible after the initial 24 h of the experiment. These conclusions were further corroborated by an estimated biomass increase of *P. bohneri* of 9·0±5·0 mg d.m. l^{-1} from day 0 to 1 calculated from previous algal culture experiments on mineral medium (Dauta, 1982), as compared to about 100 mg d.m. l^{-1} in the presence of cheese factory anaerobic effluent, and by *in situ* biomass increase of *P. bohneri* in cheese factory anaerobic effluent, from day 0 to 1, calculated from cellular chlorophyll concentration and corresponding to 8·3 mg d.m. l^{-1}. Thus, after subtracting the initial decrease in dry mass due to adhesion of the organic matter, growth rates of *P. bohneri* were estimated graphically from the slope of the exponential growth phase of a semi-log graph of biomass increase as a function of time.

Nutrient analysis

Nutrient analyses were done on the filtrate obtained during biomass determination. Samples were stored on ice during sampling and then analyzed for ammonia N-NH_3 with the Nesslerization method (APHA, 1989). Samples for P-PO_4^{3-} were acidified with 1% v/v of 30% H_2SO_4 and kept in glass bottles in the dark at 4°C before analysis within 15 days with a Technicon II autoanalyzer, according to method 94-70W/B, with the reagents ammonium molybdate and ascorbic acid. Samples for NO_2^--NO_3^- were kept at −20°C and analyzed within 30 days with the Technicon II autoanalyzer using the sulfanilamide and N.E.D. reagents method (MENVIQ., 1992, No. 87.06/303-NO_3 1.1).

RESULTS AND DISCUSSION

The growth of *P. bohneri* on diluted cheese factory effluent caused an increase in pH of 2·5 units in four days to reach a maximum value of 10·9 (Fig. 1), indicating that this medium was poorly buffered. In comparison, during the growth of *M. pusillum*, the pH increased only 0·9 units to reach a maximal value of 9·5 after four days (Fig. 1). In the effluent without algae, the pH remained more or less constant at 8·5. An increase of two pH units had also been reported during the growth of *P. bohneri* on effluent from the secondary treatment of domestic wastewater (Proulx *et al.*, 1994).

On anaerobically-treated cheese factory effluent, the growth rate of *P. bohneri* ($k = 0{\cdot}62$ d^{-1}, Table 1) was almost twice that of *M. pusillum* ($k = 0{\cdot}35$ d^{-1}, Table 1). After four days, from an initial concentra-

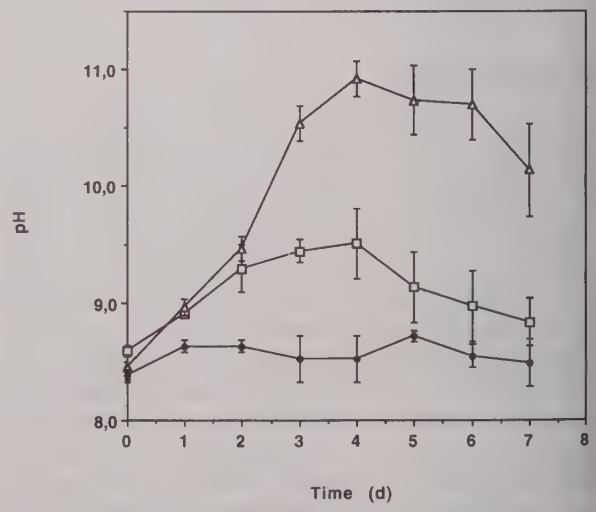

Fig. 1. The pH value, taken at 10:00 in the morning, in cheese factory anaerobic effluent without algae (●) or in the presence of *P. bohneri* (△) or *M. pusillum* (□) ($n = 4 \pm$ s.d.).

Table 1. Growth and nutrient removal in batch culture of *P. bohneri* and *M. pusillum* on cheese anaerobic effluent. The inoculum concentration was 111 mg d.m. l^{-1}

Treatment	Growth rate (k)	Biomass yield (mg d.m.) (0–4d)	Ammonia removal rate		Phosphorus removal rate	
			(mg l^{-1} d^{-1})	(mg N mg d.m.$^{-1}$ d^{-1})	(mg l^{-1} d^{-1})	(mg P mg d.m.$^{-1}$ d^{-1})
P. bohneri	0·62	329±24	5·9	0·027	2·9	0·014
M. pusillium	0·35	137±21	9·3	0·072	2·5	0·020
Effluent without algae	—	—	2·3	—	0·2	—

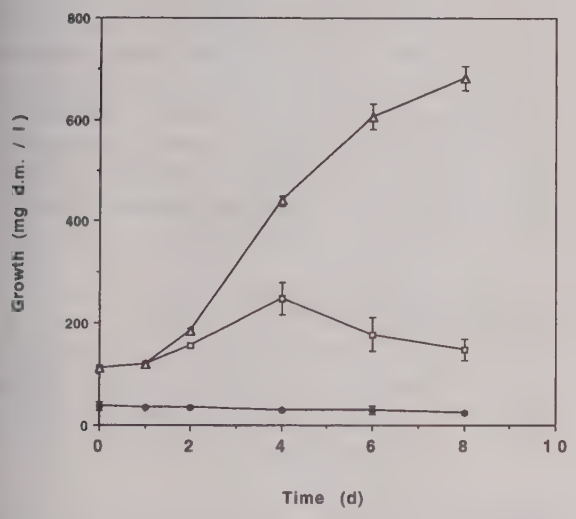

Fig. 2. Variation in cell dry mass in cheese factory anaerobic effluent without algae (●) or in the presence of *P. bohneri* (△) or *M. pusillum* (□) ($n = 4 \pm$ s.d.). When not visible, the error bar is included within the symbol.

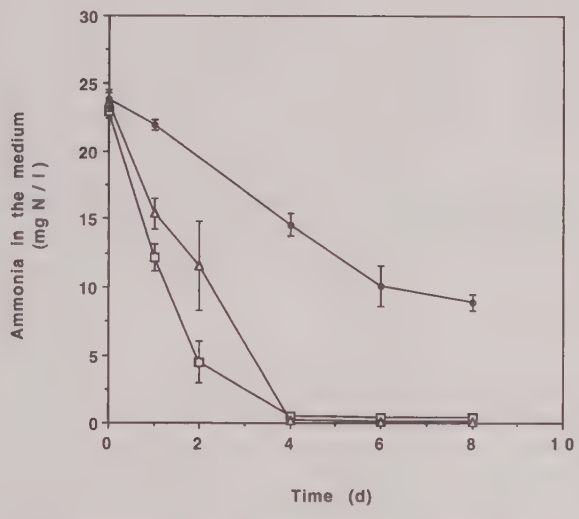

Fig. 3. Ammonia concentration in cheese factory anaerobic effluent without algae (●) or in the presence of *P. bohneri* (△) or *M. pusillum* (□) ($n = 4 \pm$ s.d.).

tion of 111 mg d.m. l^{-1}, *P. bohneri* reached a biomass of 440 mg d.m. l^{-1}, as compared to 248 mg d.m. l^{-1} for *M. pusillum* (Fig. 2). During the ensuing four days, *P. bohneri* grew an additional 240 mg to reach 680 mg d.m. l^{-1} on the eighth day. These data are in good agreement with the growth rate of 0·5 d^{-1} and the biomass production of about 600 mg d.m. l^{-1} over a one week period reported during the growth of *P. bohneri* on effluents from the secondary treatment of domestic wastewater (Talbot & de la Noüe, 1993).

Biomass productivities of 82 and 11 mg d.m. l^{-1} d^{-1} were calculated, during the initial four days, for *P. bohneri* and *M. pusillum*, respectively. While a high rate of aeration favors the maintenance of flocs of *P. bohneri* in suspension, it may have inhibited the growth of *M. pusillum*. Azov *et al.* (1980) reported that high shear forces generated by strong aeration break the spines (setae) of *M. pusillum* and are thus detrimental to its growth. Microscopic observations confirmed such damage to *M. pusillum* cells during our experiments. *Micractinium pusillum* cell mass decreased between days 4 and 8, to stabilize at 148 mg d.m. l^{-1} (Fig. 2). This decrease in *M. pusillum* biomass following the experimental growth phase was due to adhesion of the cells to the sides of the culture vessels, even in the presence of strong aeration. Similarly, the decrease in suspended solids, observed at a rate of 1·5 mg l^{-1} d^{-1}, in the effluent without algae was due to their adhesion to the sides of the culture vessels ensuing in the formation of a biofilm.

No significant amount of ammonia was left in the medium after four days of growth in the presence of *P. bohneri* or *M. pusillum* (Fig. 3). While ammonia was removed at a fairly constant rate of 5·9 mg N-NH_3 l^{-1} d^{-1} or 0·027 mg N-NH_3 mg d.m.$^{-1}$ d^{-1} by *P. bohneri* (Fig. 3), a high initial rate of removal was observed with *M. pusillum* culture during the first two days (9·3 mg N-NH_3 l^{-1} d^{-1} or 0·072 mg N-NH_3 mg d.m.$^{-1}$ d^{-1}), followed by a decrease. The control without algae showed a removal rate of 2·3 mg N-NH_3 l^{-1} d^{-1}, resulting in a 57% reduction in ammonia after six days (Fig. 3). This removal was probably due to the stripping of ammonia and to bacterial activity. Although no quantification of ammonia stripping was made, this could certainly be an important mechanism for ammonia loss, especially in the presence of *P. bohneri* and *M. pusillum*, where the pH reached values of 10·9 and 9·5, respectively. Estimation of stripping using a control culture leads to underestimated values as pH values in the absence of microalgae do not exceed 8·5. Talbot and

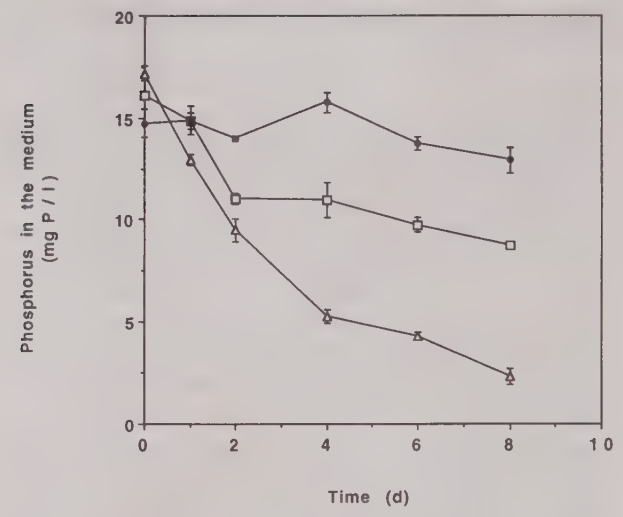

Fig. 4. Orthophosphate concentration in cheese factory anaerobic effluent without algae (●) or in the presence of *P. bohneri* (△) or *M. pusillum* (□) ($n = 4 \pm$ s.d.).

de la Noüe (1993) reported 38% ammonia removal by stripping in cultures of *P. bohneri* on domestic effluent at 30°C with maximal pH values of 9·2. Throughout the experiment, total nitrite and nitrate concentrations were negligible with values below 0·1 mg NO_2^--NO_3^- l^{-1}.

The removal rate of phosphorus was fairly constant during the initial four days in the presence of *P. bohneri* (2·9 mg P-PO_4^{3-} l^{-1} d^{-1} or 0·014 mg P-PO_4^{3-} mg d.m.$^{-1}$ d^{-1}), while most of the phosphate removal occurred during the initial two days in the presence of *M. pusillum* at a rate of 2·5 mg P-PO_4^{3-} l^{-1} d^{-1} or 0·020 mg P-PO_4^{3-} mg d.m.$^{-1}$ d^{-1} (Fig. 4). However, after four days of culture, only 33% of the total phosphorus disappeared from solution with *Micractinium*, as compared to 69% with *P. bohneri*. In the effluent without algae, phosphorus concentration remained relatively constant at a level of 14·3 mg P-PO_4^{3-} l^{-1}.

The increase in pH observed during the growth of *P. bohneri* and *M. pusillum* is certainly one of the key factors influencing the removal of ammonia and phosphorus in cheese factory effluent. In fact, during the initial four days of the experiment, a significant correlation was observed between pH values and the amount of ammonia and phosphate left in the medium with correlation coefficients (r) always above 0·962. High pH values favor the removal of ammonia via stripping (Idelovitch & Mitchail, 1981) and the removal of phosphorus by precipitation following the formation of hydroxyapatite (Diamadopoulos & Benedek, 1984). Doran and Boyle (1979) reported that over 90% of the phosphorus removal of an activated algae process was achieved by means of chemical precipitation.

It is certain that the capacities of removal of inorganic nutrients by *P. bohneri* and *M. pusillum* in cheese factory effluent were influenced by the presence of suspended organic matter (36 mg l^{-1}). In fact, visual observations showed that organic matter was disadvantageous to *Micractinium* by causing cell clogging and adhesion to the walls of the culture vessels. In contrast, adhesion of organic matter to the flocs of *P. bohneri* was effective in reducing the suspended solids from the effluent in parallel with removal of inorganic nutrients.

Finally, the fact that *Phormidium bohneri* demonstrated better growth and nutrients removal capacity than *M. pusillum* on cheese factory anaerobic effluent indicates the potential of this cyanobacterium for tertiary wastewater treatment. In addition, knowing that the cyanobacterium *Anacystis nidulans* can grow well in 60% v/v dairy effluent (Thangaraj & Kulandaivelu, 1994) it will be of interest to study the capacity of *P. bohneri* to remove inorganic nutrients from more concentrated effluents.

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EFFECT OF ORGANIC LOADING RATE ON ANAEROBIC TREATMENT OF SLAUGHTERHOUSE WASTEWATER IN A FLUIDISED-BED REACTOR

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Abstract

COD removal efficiencies in the range 75·0–98·9% were achieved in an anaerobic fluidised-bed reactor treating slaughterhouse wastewater, when evaluated at organic loading rates (OLR) of between 2·9 and 54·0 g COD/l.d, hydraulic retention times (HRT) of between 0·5 and 8 h and feed COD concentrations of between 250 and 4500 mg/l. More than 94% of feed COD could be removed up to OLR of about 27 g COD/l.d. Up to 0·320 litres of methane were produced per gram of COD removed and this methane production rate was independent of the OLR applied in this investigation. Volatile fatty acid (VFA) concentration in the reactor increased sharply at an OLR of about 30 g COD/l.d and, therefore, sufficient alkalinity should be provided to prevent pH from dropping to an undesirable level. The anaerobic fluidised-bed system can be operated at a significantly higher liquid throughput than other previously reported systems while maintaining its excellent efficiency.

Key words: Anaerobic fluidised-bed reactor, slaughterhouse wastewater, substrate conversion, gas production, organic loading rates.

INTRODUCTION

Food indutry wastewaters can advantageously be treated in anaerobic reactors containing high levels of methanogenic biomass (Jeris, 1983). Wastewaters from slaughterhouses fall into this category but, traditionally, aerobic treatment processes have been applied (Gariepy *et al.*, 1989; Chudoba & Hruby, 1989). Conventional digesters, where used (Suhrkamp & Jannsen, 1989; Tritt, 1992*a*), have been shown to give only low rates of organic matter removal and to require long hydraulic retention times and thus large reactor volumes. They are also reported as being sensitive to shock loads, which is a serious disadvantage. Recent developments in anaerobic treatment technology have overcome many of these previous objections (Bull *et al.*, 1984) and have included: phase separation of the anaerobic process (Stephenson & Lester, 1986); the adoption of upflow anaerobic sludge blanket (UASB) reactors (Sayed *et al.*, 1984; Sayed & De Zeeuw, 1988); the development of fixed-film systems such as anaerobic filters (Li *et al.*, 1984; Campos *et al.*, 1986; Metzner & Temper 1990; Tritt, 1992*b*) and fluidised-bed reactors (Bull *et al.*, 1983; 1984; Rudd *et al.*, 1985).

Previous studies have shown the feasibility of using the UASB process for a one-stage anaerobic treatment of slaughterhouse wastewater. Sayed *et al.* (1984) described the performance of an UASB process for treatment of a waste, containing approximately 50% insoluble suspended COD, in a 30 m^3 pilot plant operating at 30°C. The process started up at an organic loading rate (OLR) of 1 g COD/l.d (biomass load, 0·11 g COD/g VSS.d) and a hydraulic retention time (HRT) of 35 h and could satisfactorily handle loading rates less than 3·5 g COD/l.d at a HRT of 8 h. A treatment efficiency of around 70%, on a COD basis, was achieved with a methane yield of 0·28 l/g COD removed. More recently the same research group (Sayed *et al.*, 1987; Sayed & De Zeeuw, 1988) showed, with laboratory-

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scale UASB reactors, that loading rates up to 11 g COD/l.d could satisfactorily be treated at a process temperature of 30°C and 7 g COD/l.d at 20°C; treatment efficiency ranged between 55 and 85% with up to 82% conversion of soluble materials into methane. Other studies (Zhang & Wu, 1984) have shown that this type of reactor can remove COD and coliform bacteria with efficiencies of 77–83% and 99% respectively, at loading rates of 4·7–9·4 g COD/l.d, HRT 4·7–11·5 h and 35°C temperature.

Campos *et al.* (1986) described the performance of an anaerobic filter, with gravel as the support medium, over a period of 6 years and showed COD removal efficiencies of around 80% at an organic loading of 1·4 g COD/l.d and a HRT of 13 h. Tritt (1992*b*) showed two filters, using bamboo and bones as carriers, could be operated at loading rates between 2 and 18·5 g COD/l.d and a HRT between 0·5 and 5 days; in this case COD reductions were between 30 and 85%.

This paper reports and discusses a laboratory investigation on the performance evaluation of an anaerobic fluidised-bed reactor treating slaughterhouse wastewater, with emphasis placed on the evaluation of substrate utilisation and gas-production rates under different operating conditions.

METHODS

Equipment

The fluidised-bed reactor consisted essentially of conical-shaped acrylic vessel with total and working volumes of 1·2 and 1 l respectively. The reactor column itself had a height of 50 cm with an increase in diameter from 4 cm at the base to 7 cm at the top, and attached to this was an upper settling section which was 6 cm high and 12 cm diameter. Fifty grams of clay particles (betonite), which occupied a static bed volume of 50 ml, were used as a biomass carrier, the reactor working volume being the sum of the volume of clay plus liquid. Effluent was recycled from the settlement zone to the bottom of the reactor using a recycle pump operated at a constant rate of 40 l/h, enough to provide complete fluidisation of the clay. This recycle rate created essentially well-mixed conditions in the reactor. The settlement zone also contained a conical, sheet-steel, gas–liquid separator to allow venting of the biogas produced. Four equally-spaced sampling ports were installed along the column length to obtain bed samples. Influent was pumped in continuously at the bottom of the reactor by means of a peristaltic pump and effluent was withdrawn from the top; the level in the reactor was controlled by a liquid overflow.

Biogas produced from the reactor was collected by positive displacement of acidified water (pH 2–3) in 5-litre gasometers. The operating temperature of the reactor, 35°C, was maintained as constant by means of an external water jacket through which water from a thermostatic bath circulated.

Support material

Clay particles of bentonite of 0·3–0·5 mm diameter were used as the growth-support material. The main characteristics of this packing medium were: porosity, 63%; specific surface area, 250 m^2/g; bulk density, 0·9 g/ml; wet density, 1·52 g/ml; cation-exchange capacity, 125–140 mequiv./100 g. It was selected because of its ability to readily attach methanogenic bacteria (Pérez *et al.*, 1989) and its promotion of favourable kinetic behaviour in reactors treating this type of wastewater (Borja *et al.*, 1993). A detailed description of the composition and features of this packing medium is given elsewhere (Borja *et al.*, 1992).

Wastewater

The wastewater was collected from an abbatoir which slaughters about 70% cattle and 30% hogs and produces a daily flow of 150 m^3. The wastewater was collected after sieving in the same factory to remove coarse solids. The features and composition of this wastewater are summarised in Table 1, which lists the average values of five separate analyses for each parameter; there was virtually no variation (less than 1%) between analyses.

Seed preparation

The sludge taken from an industrial anaerobic reactor treating municipal wastewater was collected and screened through a sieve to remove coarse materials. Then 1 l of screened sludge was transferred to a laboratory-scale, batch-type, anaerobic digester maintained at 35°C. Diluted slaughterhouse wastewater (50 ml) of 2500 mg COD/l was added to this digester each day for 10 consecutive days to obtain 1·5 l digested sludge. Then 75 ml sludge was removed each day from the digester and replaced with another 75 ml of diluted wastewater (COD 2500 mg/l) to maintain a constant sludge volume. This practice was continued for about 2 months to ensure that the seeding sludge was fully acclimatised to the wastewater used in this investigation. The methanogenic activity of this sludge was 0·29 g COD/g VSS.day.

Table 1. Composition and features of the wastewater

pH	6·7
COD	5·05 g/l
BOD_5	3·12 g/l
Total suspended solids (TSS)	0·10 g/l
Volatile suspended solids (VSS)	0·07 g/l
Volatile acidity (as acetic acid)	0·12 g/l
Alkalinity (as $CaCO_3$)	0·41 g/l
Phosphorus (PO_4^{-3})	0·03 g/l
Ammoniacal nitrogen	0·095 g/l
Kjeldahl nitrogen	0·31 g/l
Protein	1·85 g/l

Start-up of the anaerobic fluidised-bed reactor

The reactor was initially filled with 50 g of bentonite clay particles, then 500 ml of acclimatised seeding sludge was added to the reactor and it was filled with 450 ml of tap water. Then the reactor was operated in a total recycle fashion with initial bed expansion maintained at 40%. Sodium bicarbonate was added if necessary to maintain the reactor pH in the range of 6·8–7·2. Diluted slaughterhouse wastewater (100 ml) of 2500 mg COD/l was added to the reactor each day to promote and sustain the growth of biofilms on the clay particles. After 2 months of operation in this fashion to ensure a complete adaptation to the wastewater used, bentonite particles in the reactor were covered with biofilms, as could be seen by scanning electronic micrographs.

Experimental procedure

After the start-up period, the steady-state experiments were performed by varying the feed COD concentration and the HRT. The following six feed COD concentrations were chosen: 250, 500, 1000, 2000, 3000 and 4500 mg/l. At a given feed COD concentration, the reactor was operated at a predetermined feed-rate to attain required HRT and OLR. With this experimental design, the HRT were varied in the range of 0·4–0·6 h (feed COD = 250 mg/l), 0·8–4·1 h (feed COD = 500 mg/l), 1–6 h (feed COD = 1000 mg/l), 1·5–8·0 h (feed COD = 2000 mg/l) and 2–8 h (feed COD = 3000 and 4500 mg/l) and the OLR applied (based on the reactor working volume) was varied in the range of 2·9 to 54·0 g COD/l.d.

In each experimental run, the reactor was continuously operated under the above-mentioned feed concentrations and loading rates until steady-state conditions were reached; i.e. effluent COD and methane production-rate remained relatively constant. Then samples were collected and analysed for at least 5 consecutive days. The steady-state value of a given parameter was taken as the average of these consecutive measurements for that parameter when the deviations between the observed values were less than 3% in all cases.

The organic loadings applied in this investigation were increased in a stepwise fashion in order to minimise the transient impact on the reactor that might be induced by a sudden increase in loadings.

Chemical analyses

The following parameters were analysed according to Standard Methods (APHA, 1989): COD (determined on filtered samples), BOD_5, pH, total suspended solids (TSS), volatile suspended solids (VSS), alkalinity, phosphorus (PO_4^{3-}), ammoniacal nitrogen, Kjeldahl nitrogen and protein.

Methane was determined by gas chromatography with a stainless-steel column (200 × 0·3 cm) packed with active carbon (30–60 mesh) using thermal conductivity detection.

Volatile fatty acids were determined by gas chromatography using a 2 m × 4 mm glass column packed with Supelcopor (100–200 mesh) coated with 10% Fluorad FC 431. The temperature of the column, the injection port and the flame ionisation detector were 130, 220 and 240°C respectively. Nitrogen saturated with formic acid was used as the carrier gas at a flow-rate of 50 ml/min.

Biomass concentration was estimated at the end of each series of experiments at constant feed COD concentration according to the recommendations of Chen *et al.* (1985) by taking out a small volume of the reactor liquid (about 15 ml of mixed liquid).

In each steady-state experiment, samples were collected and the above parameters analysed. The pH, alkalinity and gas volume and composition were determined daily, whilst the remaining parameters were measured at least five times per week on five different samples taken on different days to ensure that representative data were obtained.

RESULTS AND DISCUSSION

Effects of organic loading rate on the substrate utilisation in the reactor

The COD removal efficiencies observed at different OLR are illustrated in Fig. 1. In general, the percentage of COD removed decreased linearly with increased OLR over the range applied in this investigation. More than 85% of feed COD could be removed up to OLR of about 35 g COD/l.d, which indicates that the anaerobic fluidised-bed system is highly effective.

The organic removal rates observed at different OLR are illustrated in Fig. 2. Although the COD removal efficiency of the system decreased linearly with increased OLR, the system was capable of

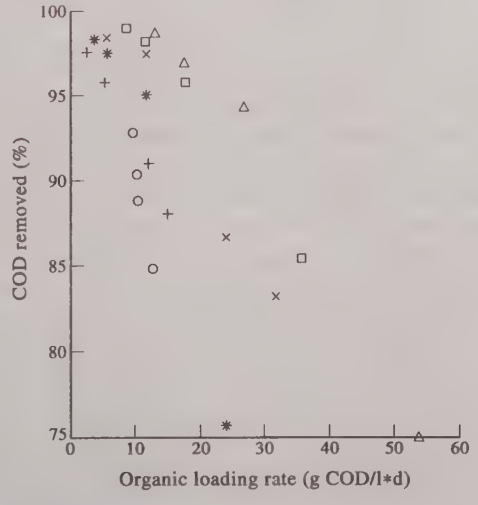

Fig. 1. Variation in the percentage of COD removal with the organic loading rate (g COD/l.d) of the reactor (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

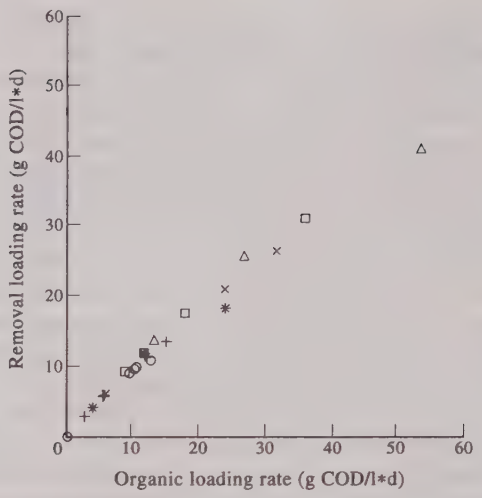

Fig. 2. Variation of the removal loading rate (g COD/l.d) with the organic loading rate (g COD/l.d) of the reactor (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

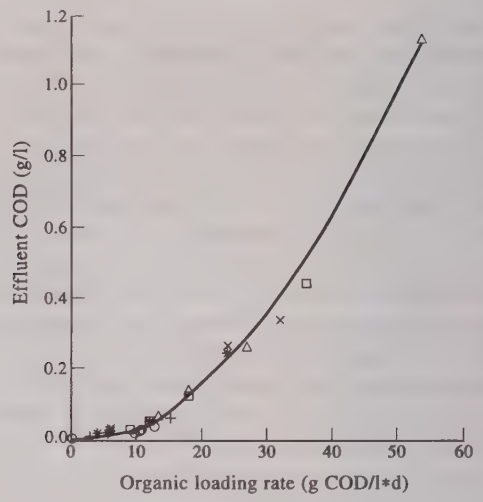

Fig. 3. Variation of the effluent COD (g/l) with the organic loading rate (g COD/l.d) of the reactor (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

removing more substrate at higher loadings. The attainment of high reactor biomass hold-up in the anaerobic fluidised-bed system, via the immobilisation of microorganisms on the small, fluidised particles, contributed to such a good system-efficiency. In fact, the equilibrium biomass hold-up in the reactor was strongly dependent upon the OLR applied, the equilibrium biomass hold-up increased continuously from 12 450 mg/l at an OLR of 2·9 g COD/l.d to 28 120 mg/l at an OLR of 54 g COD/l.d. In addition, the production and subsequent release of methane from the biofilms could have had a profound effect on the equilibrium biofilm thickness (and, therefore, equilibrium biomass hold-up) in the reactor, because the resulting effervescence might have sloughed the biofilms off the clay particles. It was, therefore, expected that VSS in the effluent would increase with increasing methane production. In fact, the average VSS concentration in the effluents increased from 150 to 410 mg/l when the feed COD concentration increased from 250 to 4500 mg/l. Because the wastewater used contained almost entirely soluble constituents (Table 1), all VSS measured in the effluent could be considered as biological solids.

The effluent COD of the anaerobic fluidised-bed reactor increased with increased OLR, as illustrated in Fig. 3. Such an increase in the effluent COD was paralleled by a similar increase in the effluent volatile acids (VFA), as illustrated in Fig. 4. This seems to indicate that, at higher OLR, the effluent COD is largely composed of the unused volatile acids produced in the reactor.

Because the buffering capacity of the experimental system was maintained at favourable levels with excessive total alkalinity present at all loadings, the rate of methanogenesis was not affected. The

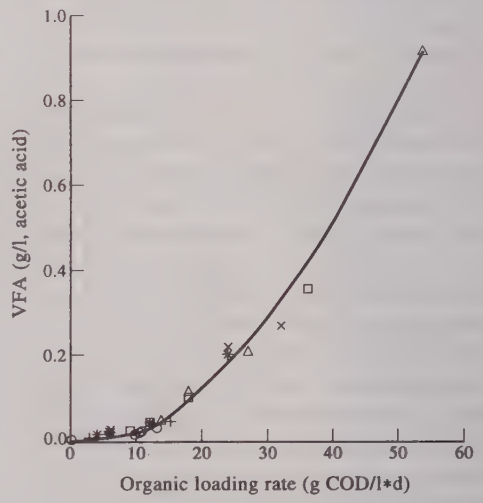

Fig. 4. Variation of the volatile fatty acid (VFA) concentration (g/l, acetic acid) with the organic loading rate (g COD/l.d) of the reactor (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

experimental data obtained in this investigation indicate that a total alkalinity of about 2500 mg/l as $CaCO_3$ is sufficient to prevent the pH from dropping to below 7·0 at an OLR of 54 g COD/l.d.

The COD removal efficiencies observed at different HRT are illustrated in Fig. 5. It appears that the performance of the anaerobic fluidised-bed system becomes independent of HRT, provided that the HRT of the reactor is maintained above 2 h. Below that the performance of the reactor deteriorates sharply. It is also interesting to note that, at higher HRT, the performance of the reactor is independent of the feed COD concentration.

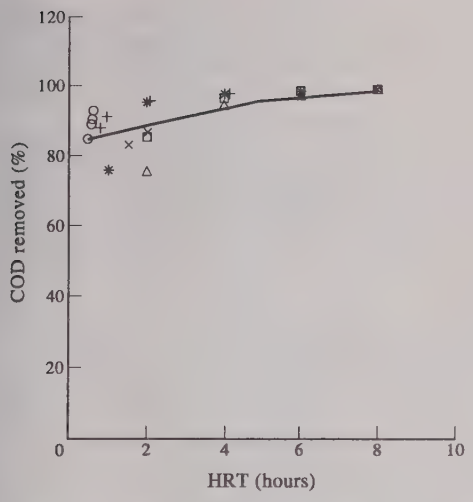

Fig. 5. Effect of HRT (h) on the percentage of COD removal for the different feed COD concentrations used (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

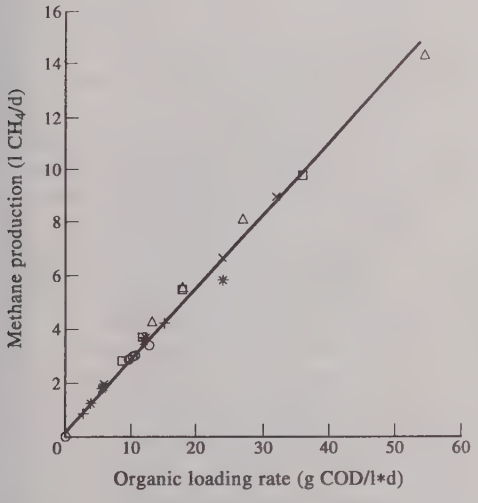

Fig. 6. Variation of the methane production rate (1 CH_4/d) with the organic loading rate (g COD/l.d) of the reactor (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

Effects of organic loading rate on the methane production in the reactor

The volumetric methane production rate as a function of OLR is illustrated in Fig. 6. It is seen that the volume of the methane produced per day increased with increased OLR over the range tested. Apparently the activity of methanogenic bacteria was not impaired at higher OLR because of the adequate buffering capacities provided in the experimental system. Higher biomass hold-ups in the reactor would also promote such high activities of methanogenesis. Nevertheless, the methane content of the gas produced decreased from 78 to 59% when the OLR was increased from 2·9 to 54 g COD/l.d.

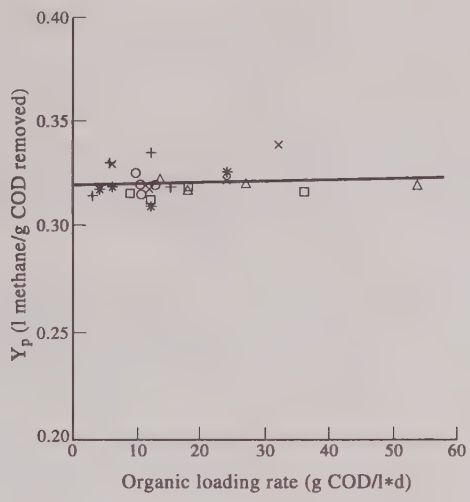

Fig. 7. Variation of the volume of methane produced per unit weight COD removed Y_p (1 CH_4/g COD) with the organic loading rate (g COD/l.d) of the reactor. (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

This decrease in the methane content with OLR might be attributed to an inhibition of the methanogeneic bacteria at high OLR values, which caused an increase in effluent VFA contents, as can be seen in Fig. 4.

The volume of methane produced per unit weight COD removed (Y_p) as a function of OLR is illustrated in Fig. 7. Up to about 0·32 l of methane were produced per g COD removed and this rate was independent of the OLR applied. Theoretically, 0·35 l of methane is produced per gram of COD removed when the starting compound is glucose (Lawrence & McCarty, 1969). The effectiveness of the anaerobic fluidised-bed reactor in converting slaughterhouse wastewater into methane is clearly demonstrated.

CONCLUSIONS

The performance of the anaerobic fluidised-bed reactor designed for the purification of slaughterhouse wastewater, when evaluated at OLR of between 2·9 and 54·0 g COD/l.d, HRT of between 0·4 and 8 h and feed COD concentrations of between 250 and 4500 mg/l gave steady-state COD removal efficiencies in the range of 75·0 to 98·9%. Under the operating conditions evaluated in this investigation a HRT higher than 2 h is recommended in order to ensure a good system-efficiency. The high performance may be attributable to the carrier material, the design of the bed, and the appropriate acclimatisation of the biomass and start-up of the reactor performed to minimise the undesirable wash-out of slow-growing methanogenic bacteria.

Because the effluent COD in the anaerobic reactor at higher OLR is largely produced by the unused volatile acids produced in the reactor, it is essential

that the buffering capacity of the system should be maintained with the presence of an adequate amount of alkalinity. The experimental data obtained in this investigation clearly indicate that the anaerobic fluidised-bed system can remove 75% of feed COD at an OLR as high as 54 g COD/l.d, provided that the total alkalinity is maintained at 2500 mg/l (as $CaCO_3$) in the reactor.

The high COD removal-efficiency as well as the high methane-production rate observed in this investigation indicate that the process intensification (i.e. an increase in process productivity while maintaining performance) achieved in the anaerobic fluidised-bed system is very impressive. Fluidisation of small particles in the anaerobic fluidised-bed system provides a vast amount of surface area for microbial attachment and growth, which in turn results in a high biomass hold-up in the reactor. Moreover, because virtually all the biomass in the anaerobic reactor is retained in biofilms grown on the fluidised particles, the possibility of biomass wash-out at higher hydraulic and/or organic loadings is dramatically reduced.

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KINETIC EVALUATION OF AN ANAEROBIC FLUIDISED-BED REACTOR TREATING SLAUGHTERHOUSE WASTEWATER

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Abstract

An anaerobic fluidised-bed reactor for purification of slaughterhouse wastewater was modelled as a continuous-flow, completely-mixed homogeneous microbial system, with the feed COD as the limiting-substrate concentration. The average microbial residence time in the reactor was defined in terms of conventional sludge-retention-time. The experimental data obtained indicated that the Michaelis–Menten expression was applicable to a description of substrate utilisation (i.e. COD removal) in the anaerobic fluidised-bed system. The maximum substrate utilisation rate, k, *and the Michaelis constant,* K_s, *were determined to be 1·2/day and 0·039 g/l. The observed biomass yield in the reactor decreased with increasing sludge-retention-time. The specific methane production rate observed was a linear function of the specific substrate-utilisation rate.*

Key words: Anaerobic digestion, slaughterhouse wastewater, fluidised-bed reactor, kinetic model, Michaelis–Menten expression.

INTRODUCTION

The anaerobic fluidised-bed system is a common process in anaerobic biotechnology which has been demonstrated in a number of studies to be highly cost-effective for liquid waste treatment, for biomass conversion and for biochemical recovery and production (Boening & Larsen, 1982; Switzembaum & Danskin, 1982; Olthof & Oleszkiewick, 1982; Speece, 1983; Norrman, 1983; Jeris, 1983; Gujer & Zehnder, 1983; Yoda *et al.*, 1987; Wheatley, 1990; Kida *et al.*, 1990; Martín *et al.*, 1993; Borja & Banks, 1994). This system enables high biomass hold-up to be attained for good system efficiency and stability, with low hydraulic-retention-time for good system economy (Chen *et al.*, 1985*a*; *b*). Unlike the conventional biofilm systems in which the growth support media are fixed in space either by gravity or by direct attachment to the reactor wall, the anaerobic fluidised-bed system retains the growth support media in suspension by drag forces exerted by the upflowing wastewater. Moreover, the distribution of retained biomass (in the form of biofilms) is relatively uniform, because of the completely-mixed conditions maintained and the continuous biofilm sloughing process, which counterbalances the accumulation of biomass due to growth. Therefore, the anaerobic fluidised-bed system can, to a first approximation, be considered as a continuous-flow, completely-mixed, homogeneous, microbial system. The presence of growth-support media in the reactor has no effect on the interpretation of biomass hold-up in the anaerobic fluidised-bed system, because the biomass hold-up can be directly measured in terms of attached volatile solids using the techniques developed by Shieh *et al.* (1981) and Chen *et al.* (1985*b*).

In a previous paper by Borja *et al.* (1995), the effectiveness of an anaerobic fluidised-bed system, with bentonite as the support medium, was evaluated for purification of slaughterhouse wastewater, at a wide range of organic loading rates (OLR), hydraulic retention times (HRT) and feed COD concentrations. It was demonstrated that more than 94% of feed COD could be removed up to OLR of, approximately, 27 g COD/l·day when the HRT was maintained above 2 h. Moreover, 0·32 l of methane were produced per g COD removed, which represent 94% of the theoretical maximum value when the starting compound is glucose. The efficacy of the

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Table 1. Summary of process kinetic equations

Equation	Remarks
(1) $U = (S_o - S_e)/\theta X$	Mass balance equation on substrate in the reactor.
(2) $U = kS_e/(K_s + S_e)$	Michaelis–Menten expression for substrate utilisation in the reactor.
(3) $\theta_c = XV/QX_e$	Definition of sludge retention time.
(4) $Y_o = Y/(1 + k_d\theta_c)$	Equation for estimation of biomass production in the reactor.
(5) $U_g = \alpha U$	Equation for estimation of specific methane production rate in the reactor.
(6) $U_g = V_g\delta_g/XV$	Definition of specific methane production rate.

Where k is the maximum substrate utilisation rate (g COD/g VSS.day); k_d is the microbial decay rate (1/day); K_s is the Michaelis constant (g COD/l); S_e is the steady-state effluent substrate concentration (g COD/l); S_o is the feed substrate concentration (g COD/l); Q is the feed rate (l/day); U is the specific substrate utilisation rate (g COD/g VSS.day); U_g is the specific methane production rate (g CH_4/g VSS.day); V is the fluidised-bed volume (l); V_g is the methane volume produced (l/day); X is the biomass hold-up (g VSS/l); X_e is the effluent VSS concentration (g/l); Y is the biomass yield; Y_o is the observed biomass yield; α is a constant; δ_g is the density of methane (g/l); θ is the hydraulic retention time (days); and θ_c is the solid retention time (days).

anaerobic fluidised-bed system for purification of this wastewater was clearly demonstrated.

A kinetic model was developed in this work for the anaerobic fluidised-bed system, with slaughterhouse wastewater as the limiting substrate. Experimental data obtained in a laboratory investigation were used to determine the relevant kinetic coefficients involved in the resulting model.

KINETIC MODEL

The model (Table 1) was based on the following assumptions: (1) all reactions occur in the fluidised-bed volume occupied by the bioparticles (biofilm-support media); (2) although this system is not strictly a true chemostat type culture, it can be described by equations relating to the chemostat; (3) the Michaelis–Menten expression is applicable to a description of substrate utilisation in the fluidised-bed volume; (4) no microorganisms are contained in the feed and, therefore, effluent volatile suspended solids (VSS) can be considered as the biomass sloughed from the reactor; (5) the specific methane-production rate (g CH_4/g VSS·day) is a linear function of the specific substrate-utilisation rate (g COD/g VSS·day) in the fluidised-bed volume; and (6) steady-state conditions prevail.

The resulting mathematical expressions of the kinetic model are summarized in Table 1.

METHODS

Equipment

A laboratory-scale fluidised-bed reactor with a working volume of 1 l was used in the experiments. A recycle rate of 40 l/h created essentially well-mixed conditions in the reactor. The reactor has been described in greater detail elsewhere (Borja *et al.*, 1995).

Biogas produced from the reactor was collected by positive displacement of acidified water (pH 2–3) into 5-l gasometers. The operating temperature of the reactor, 35°C (±1°C), was maintained by means of an external water jacket, through which water from a thermostatic bath circulated.

Table 2. Composition and features of the wastewater

pH	6·7
COD	5·05 g/l
BOD_5	3·12 g/l
Total suspended solids (TSS)	0·10 g/l
Volatile suspended solids (VSS)	0·07 g/l
Volatile acidity (as acetic acid)	0·12 g/l
Alkalinity (as $CaCO_3$)	0·41 g/l
Phosphorus (PO_4^{-3})	0·03 g/l
Ammoniacal nitrogen	0·095 g/l
Kjeldahl nitrogen	0·31 g/l
Protein	1·85 g/l

Support material

Clay particles (bentonite) of 0·3–0·5 mm diameter were used as the growth support material. A detailed description of the composition and features of this packing medium is given elsewhere (Borja *et al.*, 1992).

Wastewater

The features and composition of this wastewater are summarised in Table 2, which lists the average values of five separate analyses for each parameter; there was virtually no variation (less than 1%) between analyses.

Start-up of the anaerobic fluidised-bed reactor

The reactor was initially filled with 50 g of bentonite clay particles, then 500 ml of acclimatised seeding sludge was added to the reactor and it was filled with the same volume of tap-water. A detailed description of the seed preparation and acclimatisation phase of the biomass can be found elsewhere (Borja *et al.*, 1995). Then the reactor was operated in a total recycle fashion with initial bed-expansion maintained at 40%. Sodium bicarbonate was added

if necessary to maintain the reactor pH in the range of 6·8–7·2. Diluted slaughterhouse wastewater (100 ml) of 2500 mg COD/l was added to the reactor each day to promote and sustain the growth of biofilms on the clay particles. After 2 months of operation in this fashion, bentonite particles in the reactor were covered with biofilms, as could be seen by scanning electron micrographs.

Experimental procedure

Twenty-four steady-state experiments compared various combinations of feed COD concentrations and HRT. The following six feed COD concentrations were chosen: 0·25, 0·50, 1·00, 2·00 and 3·00, 4·50 g/l. At a given feed COD concentration, the reactor was operated at a predetermined feed-rate to attain the required HRT and OLR. With this experimental design, the OLR applied varied in the range of 2·9–54·0 g COD/l·day.

In each experimental run, the reactor was continuously operated under the above-mentioned conditions until steady-state conditions were reached, i.e. effluent COD and methane-production rate remained relatively constant. Steady-state was shown by a constant (±3%) effluent COD and methane-production rate, measured daily over a period of 20–25 days.

The organic loadings applied were increased in a stepwise fashion in order to minimise any transient impact on the reactor that might be induced by a sudden increase in loading.

Chemical analyses

The following parameters were analysed according to Standard Methods (APHA, 1989): COD, BOD_5 (biochemical oxygen demand), pH, total suspended solids (TSS), volatile suspended solids (VSS), alkalinity, phosphorus (PO_4^{3-}), ammoniacal nitrogen, Kjeldahl nitrogen and protein.

Methane, volatile fatty acids and biomass-concentration analyses were described in detail in a previous paper (Borja *et al.*, 1995).

In each steady-state experiment, samples were collected and analysed. The pH, alkalinity and gas volume and composition were determined daily, whilst the remaining parameters were measured at least five times per week to ensure that representative data were obtained.

RESULTS AND DISCUSSION

Table 1 summarises the kinetic equations that can be used to describe the anaerobic digestion process. Although the anaerobic fluidised-bed system used is not, strictly speaking, a completely-mixed chemostat type culture, this experimental design is particularly suitable for this type of investigation because, once steady-state has been reached, the specific rate of substrate uptake, U, can be readily determined by a simple mass balance, represented by

$$d(VS_e)/dt = QS_o - QS_e - UVX \quad (7)$$

At the steady-state, $d(VS_e)/dt = 0$, which allows one to obtain eqn (1).

A similar equation (1) was used by Shieh *et al.* (1985) to describe the anaerobic digestion in a fluidised-bed in synthetic wastewater containing glucose as the sole carbon source and using activated-carbon particles as the microorganism support. Multiculture systems may be desirable in view of the heterogeneous nature of the microbial population performing the various bioconversion steps involved. However, the kinetic models based on this premise necessarily involve a number of kinetic equations and coefficients, making them highly complex, as shown by reported models (Lee & Donaldson, 1984; Pavlostathis & Gossett, 1986; Pavlostathis *et al.*, 1988). Complexity does not necessarily equate to accuracy and there is still a strong case in favour of a simpler kinetic treatment based on a single-culture system. Methanogenesis is particularly suited to this approach as there is a strong holistic characteristic in the process.

Equation (1) in Table 1 allows the direct calculation of the substrate utilisation rate in the reactor based on experimental data observed. Therefore, by combining eqns (1) and (2), one is able to determine experimentally whether or not the Michaelis–Menten expression is applicable for the description of substrate utilisation in the anaerobic fluidised-bed system:

$$U = (S_o - S_e)/\theta X = kS_e/(K_s + S_e) \quad (8)$$

The observed substrate utilisation rates plotted according to eqn (8) as a function of steady-state effluent COD concentrations are illustrated in Fig. 1. It can be seen from this figure that the observed substrate utilisation rates fit the Michaelis–Menten expression, which is a hyperbolic function, quite well.

Both k and K_s can be determined by plotting $(1/U)$ as a function of $(1/S_e)$:

$$1/U = \theta X/(S_o - S_e) = (K_s/k)(1/S_e) + (1/k) \quad (9)$$

From this linearised equation, k can be calculated from the intercept on the y-axis and K_s can be calculated from the slope of the straight line, as illustrated in Fig. 2. From Fig. 2, it is estimated that $k = 1{\cdot}2 \pm 0{\cdot}2$ (standard deviation throughout the paper)/day and $K_s = 0{\cdot}039 \pm 0{\cdot}007$ g/l.

The effects of maintenance energy on the net microbial yield in a continuous-flow, completely-mixed, microbial system under steady-state conditions are accounted for by defining an observed biomass yield (Y_o), which can be obtained from the experimental data. Moreover, a mass balance for the mass of microorganisms in the reactor results in eqn (4) in Table 1, which relates Y_o to the sludge retention time (θ_c) with the true biomass yield (Y) and

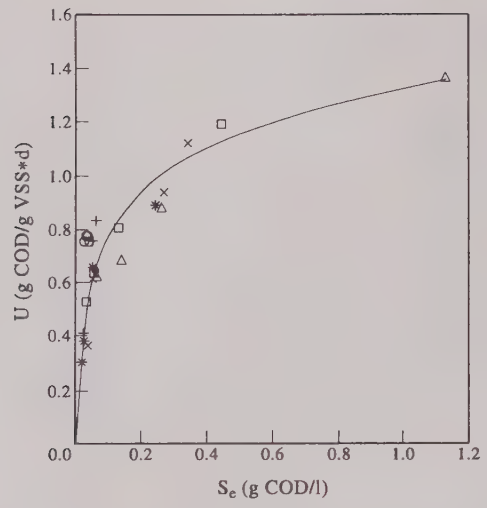

Fig. 1. The specific substrate utilisation rate (U) as a function of the steady-state effluent substrate concentrations (S_e) (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

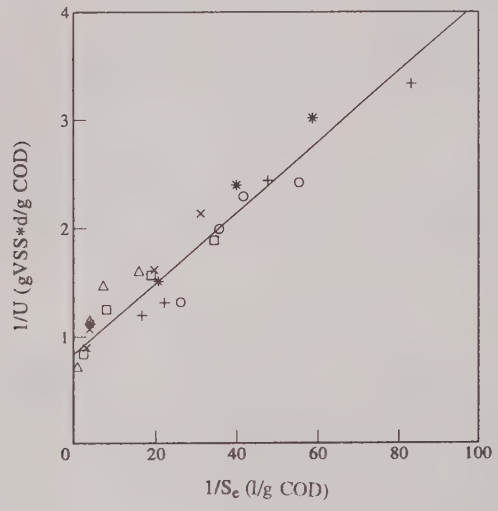

Fig. 2. The plot of ($1/U$) vs ($1/S_e$) for determination of maximum substrate utilisation rate (k) and Michaelis constant (K_s) (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

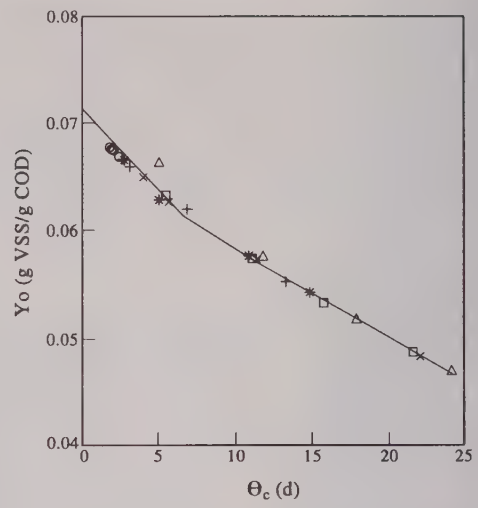

Fig. 3. The observed biomass yield (Y_o) as a function of sludge retention time (θ_c). (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

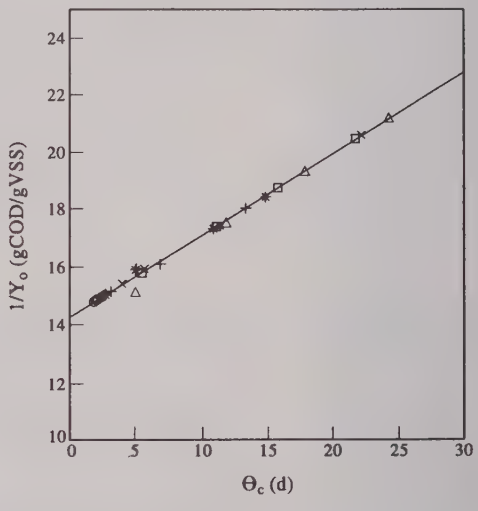

Fig. 4. The plot of ($1/Y_o$) vs sludge retention time (θ_o) for determination of true biomass yield (Y) and microbial decay rate (k_d) (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

microbial decay rate (k_d) as two intrinsic parameters. Therefore, Y_o decreases with increasing θ_c, as illustrated in Fig. 3.

Both Y and k_d can be determined by plotting ($1/Y_o$) as a function of θ_c:

$$1/Y_o = 1/Y + (k_d/Y)\theta_c \qquad (10)$$

This linearised equation is illustrated in Fig. 4 with the experimental data obtained. From Fig. 4, it is estimated that $Y = 0{\cdot}07 \pm 0{\cdot}001$ and $k_d = 0{\cdot}020 \pm 0{\cdot}003$/day. Typical values of these kinetic coefficients for the anaerobic digestion of various substrates are given in Table 3. As can be seen, the values found for this study were of the same order of magnitude as those reported in the literature (Metcalf & Eddy 1991).

The conversion of any organic substrate to methane under anaerobic conditions involves two steps. It is first converted to a mixture of short-chain organic acids with the release of carbon dioxide. Then methane is formed by either the splitting of organic acids or the reduction of carbon dioxide (Lawrence & McCarty, 1969). In a well-balanced anaerobic system under steady-state conditions, the formation rate of methane is proportional to the concentration of short-chain organic acids present. The latter, in turn, is directly related to the substrate utilisation rate that prevails. Therefore, a linear relationship

Table 3. Typical kinetic coefficients (Y, g VSS/g COD, and k_d, 1/day) for the anaerobic digestion of various substrates[a]

Substrate	Coefficient	Value	
		Range	Typical
Domestic sludge	Y	0·040–0·100	0·060
	k_d	0·020–0·040	0·030
Fatty acid	Y	0·040–0·070	0·050
	k_d	0·030–0·050	0·040
Carbohydrate	Y	0·020–0·040	0·024
	k_d	0·025–0·035	0·030
Protein	Y	0·050–0·090	0·075
	k_d	0·010–0·020	0·014

[a]Values are reported at 30°C.

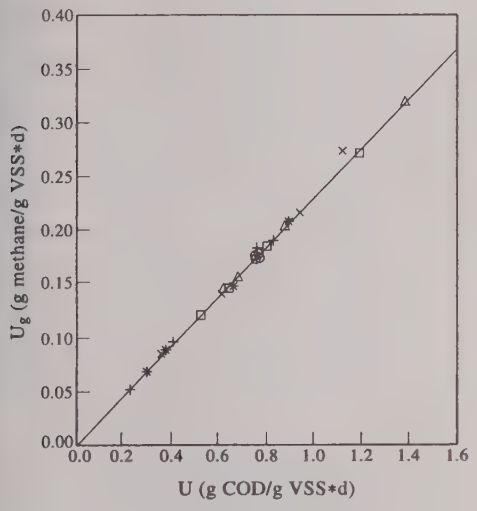

Fig. 5. The plot of the specific methane production rate (U_g) vs. the specific substrate utilisation rate (U) (○, feed COD = 250 mg/l; +, feed COD = 500 mg/l; *, feed COD = 1000 mg/l; ×, feed COD = 2000 mg/l; □, feed COD = 3000 mg/l; △, feed COD = 4500 mg/l).

exists between the formation rate of methane and the substrate utilisation rate, as illustrated in Fig. 5, with the slope of the straight line (α) as an indication of the efficiency of the system in converting glucose into methane. The α value obtained in this investigation (0·23) is about 93% of the maximum theoretical value (0·25) (Lawrence & McCarty, 1969).

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SOLVENT-FREE WOOD ESTERIFICATION WITH FATTY ACID CHLORIDES

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Abstract

A method for wood esterification using fatty acid chlorides in the absence of organic solvents is proposed. A nitrogen stream was used to carry-off the hydrogen chloride formed during synthesis and to displace the equilibrium of the reaction. The experimental conditions being optimal (nitrogen flow rate, temperature and reaction time, quantity of fatty acid chloride), there was an increase in weight of 87% and a 60% ester content for the sample esterified with octanoyl chloride. The generalization of the reaction to other fatty acid chlorides was studied. Improvement in the thermoplastic properties of wood following esterification was demonstrated.

Key words: Wood, esterification, solvent-free reaction, thermoplastic properties.

INTRODUCTION

There are many by-products (wood shavings, sawdust, etc.) in the wood-processing industry. It is possible to enhance the value of these by-products by transforming them into either thermosetting (Matsuda, 1992; Nouwezem *et al*., 1993) or thermoplastic (Shiraishi, 1991; Funakoshi *et al*., 1979) materials. However, although wood is known to be an excellent natural composite material made basically of three biopolymers (cellulose, hemicelluloses and lignin), it cannot be molded because it lacks plasticity. This is due to the chemical bonds between its main components, the crystalline nature of cellulose and the tridimensional molecular structure of lignin.

Chemical modification of wood (e.g. esterification) improves its thermoplastic properties determined from its apparent melting temperature (Shiraishi, 1991). So far, all the reactions have been conducted using organic solvents (mixture of dimethylformamide and pyridine, or benzene). Esterification agents can be a fatty acid chloride (Funakoshi *et al*., 1979; Shiraishi *et al*., 1979*a*; 1982; 1983), or a fatty acid in the presence of trifluoroacetic anhydride (TFAA) (Arni *et al*., 1961; Nakano *et al*., 1986). These esterification agents must contain at least six atoms of carbon in order to improve thermoplastic properties of wood (Shiraishi *et al*., 1979*b*). For example, the apparent melting temperature of wood acetylated with acetic acid and trifluoroacetic anhydride is 320°C (under 3 kg/cm^2) and 195°C for wood esterified with lauric acid in the same conditions (Shiraishi, 1991). This value decreases when the carbon chain of the grafted radical is increased (Shiraishi *et al*., 1982; 1983).

Only Kwatra *et al*. (1992) have proposed an esterification method without solvent, but only for cellulose with palmitoyl chloride. In this case, hydrogen chloride is eliminated under a vacuum to displace the equilibrium of the reaction. We propose a similar method for wood. The different fatty acid chlorides that have been tested will act both as reagents and as solvents. As it forms, hydrogen chloride will be removed using a nitrogen stream.

METHODS

Materials

Oakwood sawdust (60–80 mesh) was used after extraction of soluble fractions using a soxhlet for a 6-h period with a 2:1 mixture of toluene and ethanol, and again for 6 h using ethanol. The sawdust was then washed with distilled water and dried at 105°C for 12 h. All the chemical reagents were supplied by the Aldrich company (France) and were used as received.

Experimental procedures

The reaction took place in a reactor having a mechanical stirrer, a condenser and a nitrogen-gas bubbling system. In order to trap the hydrogen chloride formed, a washbottle containing an aqueous sodium hydroxide solution was placed at the outlet of the condenser. Two grams of dried sawdust and 0·3 moles of fatty acid chloride were successively introduced into the reactor. Under standard condi-

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tions, the reaction was then conducted at 130°C under agitation and a continuous nitrogen stream for 4 h. The conditions were varied in the different tests. At the end of the reaction, the esterified wood was filtered and washed with ethyl ether (50 ml) and then soxhlet-extracted using ethanol for 6 h in order to remove the excess of acid chloride. Finally, the esterified wood was dried at 105°C for 12 h. Each result presented in this publication is the average of three experiments.

Characterization of the esterified wood

Gravimetric determination of the weight increase (%) (WI)

$$WI(\%)=\frac{m_1-m_0}{m_0}\times 100$$

where m_0=weight of dried initial wood sample (g); m_1=weight of functionalized wood sample (g).

Determination of the theoretical weight increase (%) (TWI)

The number of moles of hydrogen chloride formed during the synthesis was determined by acid-base titration using phenolphthalein as the indicator. Theoretical weight increase in the sample was calculated as follows:

$$TWI(\%)=\frac{n\times(M-1)}{m_0}\times 100$$

where M=molar mass of the grafted acyl radical (RCO-); n=number of moles of hydrogen chloride trapped.

Determination of the amount of ester (%) (Tangue et al., *1963)*

The mixture made up of 1 g of esterified wood (accurately weighed) and 40 ml of ethanol (75%) was heated at 55°C for 30 min and 25 ml of 0·5 N sodium hydroxide solution were then added. The mixture was again heated at 55°C for 15 min and then left to settle at room temperature for 3 days. The excess soda was measured with a 0·5 N hydrochloric acid solution with phenolphthalein as the indicator. An excess of 1 ml of acid was added and the mixture was once again left to settle for 12 h to avoid any diffusion problem. The excess of added acid was then back-titrated with a 0·5 N sodium hydroxide solution. The ester content (%) was determined as follows:

Ester content (%)

$$=[(A-B)\times N_B-(C-D)\times N_A]\times\frac{M}{10\times w}$$

where A and B=respective volumes of sodium hydroxide solution added to sample and blank (ml); N_B=normality (N) of sodium hydroxide solution; C and D=respective volumes of hydrochloric acid added to sample and blank (ml); N_A=normality (N) of hydrochloric acid solution; w=weight of sample (g); M=molar mass of grafted acyl radical.

Infra-red analysis

The infra-red spectra were recorded on a NICOLET 205 using KBr pellets.

Scanning electron microscopy (SEM)

The microscopic structures of the different sawdust samples were analysed using a scanning electronic microscope (JEOL-JSM-6400 scanning microscope).

Thermomechanical analysis

The measurements were done using a SETARAM TMA 92 thermomechanical analyser. Thirty milligrams of specimen were poured into a quartz crucible and subjected to a load of 0·39 kg/cm^2. The thermomechanical graphs were derived from studies performed at a temperature range of 30–400°C and a heating rate of 1°C/min. The softening and apparent melting temperatures were obtained from the peaks of the curves describing the sample's rate of deformation as a function of temperature.

RESULTS AND DISCUSSION

Synthesis of esterified wood

Below is the reaction studied:

$$\text{Wood}-\text{OH}+\text{CH}_3-(\text{CH}_2)_n\ {}_2-\underset{\underset{\text{O}}{\|}}{\text{C}}-\text{Cl}$$

$$\rightarrow\text{Wood}-\text{O}-\underset{\underset{\text{O}}{\|}}{\text{C}}-(\text{CH}_2)_{n-2}-\text{CH}_3+\text{HCl}$$

where n=8–18.

The extent of the esterification reaction was determined by measuring the weight increase and the amount of ester. Given that wood is chemically heterogeneous in its composition, it is impossible to calculate the degree of substitution that is generally used to describe cellulose derivatives.

Octanoyl chloride (n=8) was used as a reagent to determine how various factors affected weight increase. Without a nitrogen stream, wood deteriorated significantly (35% weight loss; Fig. 1). Above 100 ml/min, the weight increase reached a plateau. The temperature of the reaction had to be 130°C (Fig. 2) to limit the duration of the reaction to only 4 h (Fig. 3). Under these experimental conditions, 0·3 mole octanoyl chloride was necessary to ensure an 87% weight increase (Fig. 4) and a 60% ester content. Shiraishi *et al.* (1979*a*) reported 115% weight increase under very different experimental

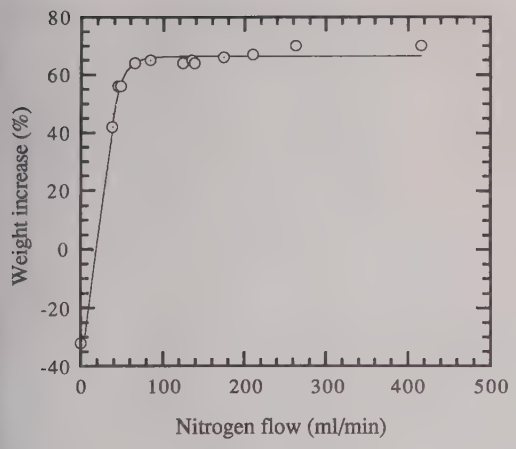

Fig. 1. Effect of nitrogen flow rate on weight increase during esterification of 2·0 g of wood with 0·3 mole of $C_8H_{15}OCl$ at 120°C for 4 h.

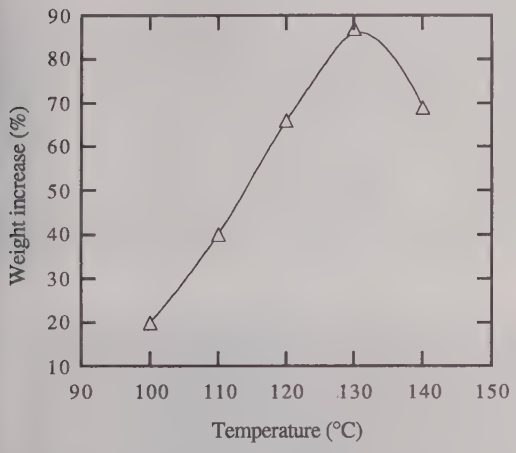

Fig. 2. Effect of temperature on the weight increase during esterification of 2·0 g of wood with 0·3 mole of $C_8H_{15}OCl$ for 4 h, nitrogen flow rate being 170 ml/min.

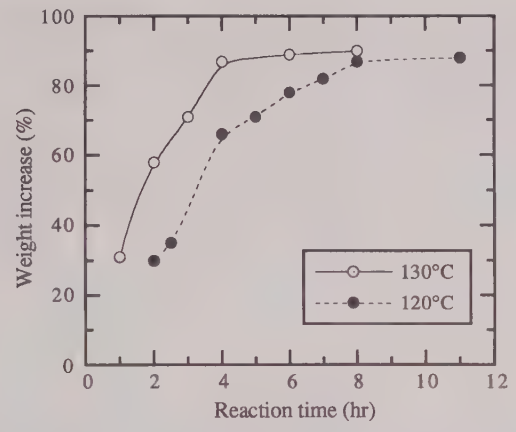

Fig. 3. Effect of reaction time on weight increase during esterification of 2·0 g of wood with 0·3 mole of $C_8H_{15}OCl$ at 120 and 130°C, nitrogen flow rate being 170 ml/min.

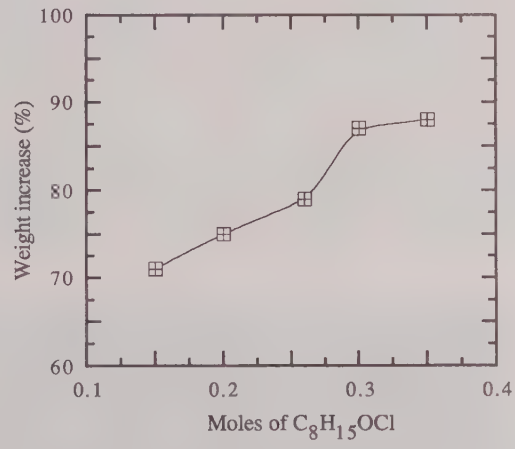

Fig. 4. Effect of the quantity of $C_8H_{15}OCl$ on weight increase during esterification of 2·0 g of wood at 130°C for 4 h, nitrogen flow rate being 170 ml/min.

conditions (N_2O_4–dimethylformamide–pyridine mixture as solvent and a reaction time of 240 h).

The infra-red analysis before and after esterification proved that a chemical change had occurred in the wood samples (Fig. 5). The intensity of the absorption band of the O–H bond vibrations decreased, whereas those of the C–H and C–O bonds increased.

However, when the value of the actual weight increase obtained (87%) is compared with the theoretical value (126%), there is a 39% difference. This is probably due to the solubilization of fractions of esterified cellulose in the octanoyl chloride, as described in the industrial process for the manufacture of cellulose acetate (Kirk & Othmer, 1979). This solubilization should also apply to esterified hemicelluloses polymers. Further studies should enable this hypothesis to be verified.

This synthesis method was applied to other acid chlorides with longer carbon chains (Fig. 6). Sig-

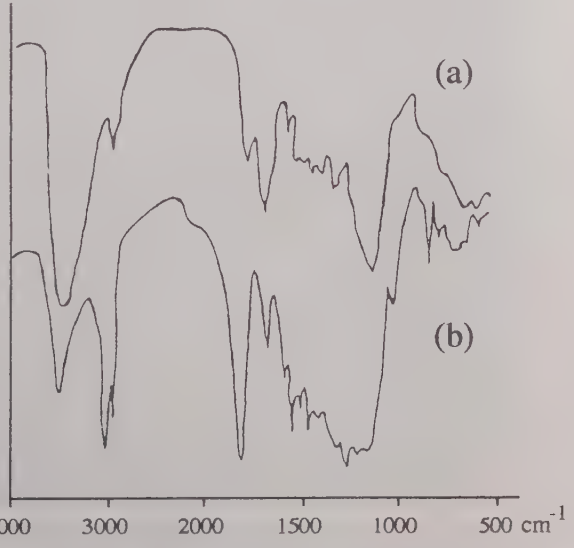

Fig. 5. Infra-red spectra of wood sawdust before (a) and after (b) esterification with octanoyl chloride.

nificantly less weight increase was obtained with an increase in the number of carbon atoms in the corresponding acyl group. This phenomenon can be the result of two factors: (1) the diffusion of the acid chloride into the wood sample is more difficult with an increase in the molecular volume of the acyl group; (2) when carbon chains are longer, the acyl group inductive effect becomes greater; therefore reactivity decreases.

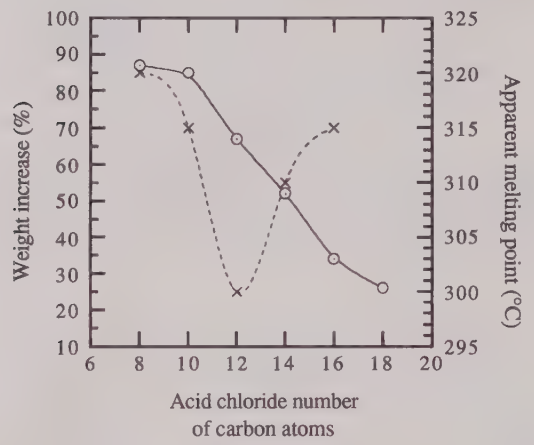

Fig. 6. Effect of nature of acid chloride on weight increase (○) and on apparent melting temperature (X) during esterification of 2·0 g of wood with 0·3 mole of acid chloride at 130°C for 4 h, nitrogen flow rate being 170 ml/min.

The change in apparent melting temperature (Fig. 6) showed that there was an improvement in thermoplastic properties compared to the non-modified wood. The thermoplasticity of chemically-modified wood is due to the creation of bulky free volume since the latter enables flexible molecular motion, such as softening or melting, under appropriate conditions (Nakano, 1994). The starting material has no apparent melting temperature. An increase in the number of carbon atoms in the grafted acyl radical logically results in a decrease in the apparent melting temperature of the esterified specimen, as is observed up to wood laurate. Beyond this, the weight increase is too small to observe this decrease in apparent melting temperature since the introduction of a small quantity of side-chains into the wood has not formed enough free volume.

The improvement in thermoplastic properties was confirmed by comparing micrographs of wood specimens before and after esterification with octanoyl chloride (Fig. 7). Pressed and non-pressed esterified wood powders had a more compact, less fibrillar, appearance.

These results prove that it is possible to esterify wood sawdust to different extents with various fatty acid chlorides in the absence of organic solvents.

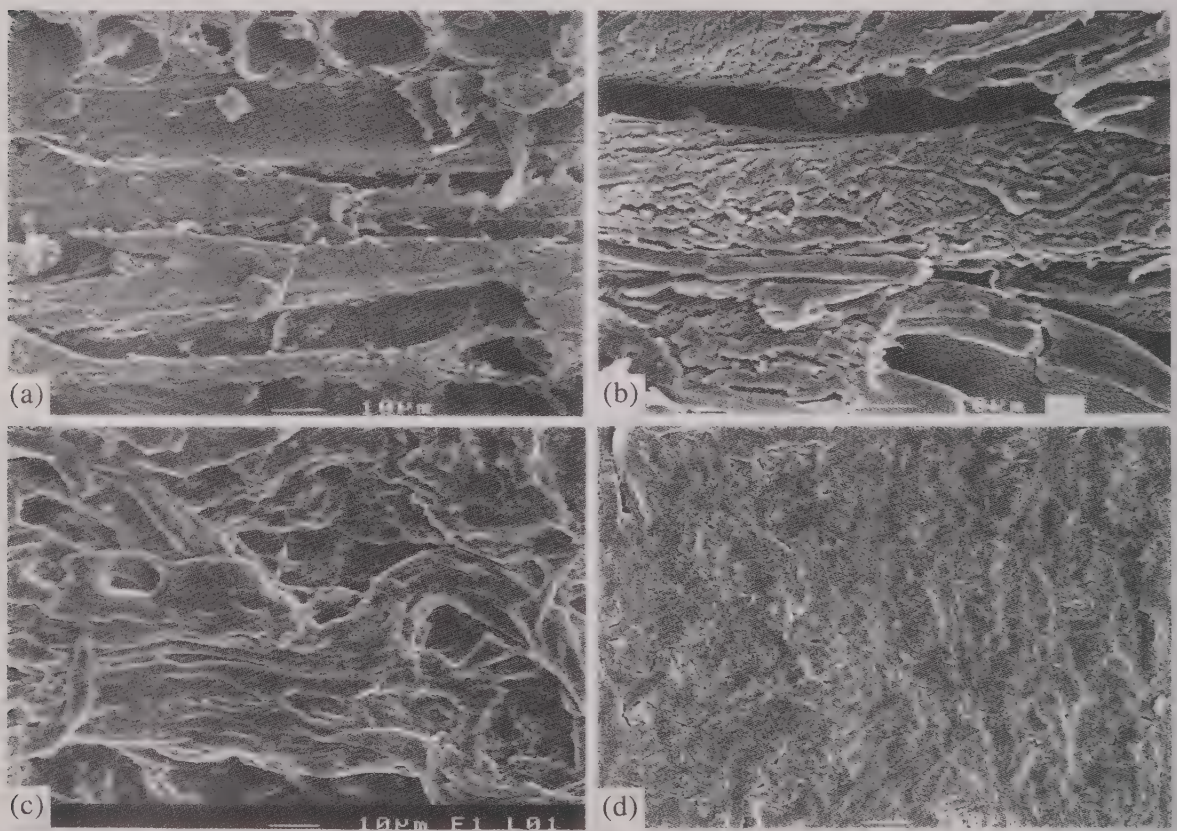

Fig. 7. Micrographs of wood samples: (a) non-esterified, powder form; (b) esterified with octanoyl chloride, powder form; (c) non-esterified film pressed at 170°C, 150 kg/cm^2, 15 min; (d) esterified with octanoyl chloride, film pressed under the same conditions.

Although the measured weight increases are lower than in the presence of a solvent (Shiraishi *et al.*, 1979*a*), the advantage of the present method is that it is simpler to use and more economical. Moreover, it facilitates the treatment of the reaction medium to recover the products.

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MICROBIAL BIOMASS AND ACTIVITY IN A GRASSLAND SOIL AMENDED WITH DIFFERENT APPLICATION RATES OF SILAGE EFFLUENT — A LABORATORY STUDY

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Abstract

This laboratory study examines the impact of different application rates of silage effluent on the biomass and activity of microorganisms in a typical grassland soil (Denbigh series). Application rates ranged from an equivalent of 0–5 l m^2 silage effluent, and amended soils were incubated at 25°C for 32 days and periodically sampled (days 2, 4, 8, 16 and 32) for microbial biomass C, CO_2 evolution and activities of various soil enzymes. After 2 and 4 days of incubation, there was a significant trend of increasing microbial biomass C ($P<0{\cdot}001$ and $P<0{\cdot}01$, respectively), microbial respiration (CO_2 evolution) ($P<0{\cdot}001$), and dehydrogenase activity ($P<0{\cdot}01$) with increasing rates of silage effluent application. Thereafter, microbial biomass C, microbial respiration and dehydrogenase activity declined and were not significantly different from the unamended control soil. Specific respiration (respired C:biomass C) increased with increasing silage effluent application, suggesting that microorganisms in amended soils may be physiologically stressed or that there is an increase in the proportion of effluent-derived bacteria relative to fungi in the amended soil. In a second experiment, there was a significant ($r^2=0{\cdot}98$; $P<0{\cdot}001$) positive linear relationship between increasing rates of silage effluent application up to 30 l m^2 and microbial respiration. Dehydrogenase activity increased up to an equivalent application of 15 l m^2 and then declined, perhaps because high concentrations of certain chemical compounds with silage effluent have an adverse abiotic impact on the enzyme assay itself. The application of different rates of silage effluent had no effect on soil pH.

Key words: Soil, grassland, microbial biomass, microbial respiration, silage effluent, enzyme activities, soil pollution.

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INTRODUCTION

Environmental pollution from farm effluents and wastes is one of the most urgent and intractable problems of livestock farming. Amongst the effluents produced on farms, the liquid that flows from ensiled grass (silage effluent) is potentially the most serious pollutant. Silage effluent is acidic fluid (approximately pH 4) and has a high BOD (13000–80000 mg l^{-1}) which can be up to 200 times higher than raw domestic sewage (IGER, 1992). In the UK, approximately 1500 million litres of silage effluent are produced each year (Brownlie & Henderson, 1984) where it can provide a valuable source of nutrients for plant growth (Purves & McDonald, 1963; Gibbs, 1977; Haland, 1979). However, indiscriminate application of silage effluent to grassland can cause scorching of grass, particularly during hot and dry weather (ADAS, 1984; Harkness, 1986).

Despite the potential toxicity of silage effluent, surprisingly little is known of its effect on soil properties and, in particular, its impact on microbial populations and their activities. The few studies that have been conducted suggest that silage effluent has no inhibitory effect on soil microbial activity, measured as CO_2 evolution (Cooper, 1977; Jones, 1986). However, there is no information on the impact of silage effluent on the abundance of soil microorganisms or on the activities of soil enzymes. There is a suggestion that because of its high BOD, rapid decomposition of effluent by soil microbes may induce oxygen depletion in the surface soil, thereby inhibiting aerobic microbial activity (Jansson, 1958; Cooper, 1977; Kuntzel, 1991). The acidic nature and toxicity of chemical components (e.g. volatile fatty acids) of silage effluent are also likely to have a detrimental effect on soil microbial populations. Inhibition of aerobic microbial activity is likely to have a profound influence on rates of organic matter decomposition and nutrient turnover and, hence, the long-term productivity of grassland. Changes in microbial biomass are often used as an early and

highly-sensitive indicator of the side-effects of various agricultural management practices (Wardle *et al.*, 1993).

The objective of the present study was to examine, under laboratory conditions, the temporal impact of different rates of silage effluent application on both the abundance and activity of microorganisms in a grassland soil. Rates of silage effluent application ranged from a low value, in line with current recommendations (1·7 l m^{-2}), to a very high value (4 l m^{-2}). An additional single-sample time experiment was conducted to examine the effects of increasing rates of silage effluent application (0–30 l m^{-2}) on soil microbial activity. Unlike other studies (e.g. Cooper, 1977), microbial activity was measured using a range of enzyme assays, as well as the more commonly used evolution of CO_2. In addition, the ratio of respired C:biomass C (metabolic quotient) was used as a measure of the physiological state of microorganisms in amended soils (Bardgett & Saggar, 1994).

METHODS

Site, soil and silage effluent

The soil used for this work was from the surface 15 cm (Ap horizon) of a clay loam, typical brown earth (Denbigh series) under a permanent, white clover–ryegrass pasture (*Trifolium repens–Lolium perenne*) at Trawsgoed, Wales (SN 677739). The soil has an average pH of 6·4 (H_2O 1:2·5 w/v), an organic carbon content of 4·4%, and a bulk density of 1·19 g cm^{-3}. The soil was chosen to represent an important high-producing grassland soil in the dairy farming areas of the western United Kingdom. The soil was sampled in June 1993 and sieved (<6 mm), mixed and stored at 4°C until use. The silage effluent used in the study was collected from a storage tank beneath a clamp of second-cut (July, 1993) ensiled grass (*L. perenne*) and had the following chemical characteristics: pH 4·0; soluble carbohydrate 9·5 mg ml^{-1}; N 0·65 mg ml^{-1}; P 0·18 mg ml^{-1}; succinic acid 2·67 mg ml^{-1}; lactic acid 1·75 mg ml^{-1}; acetic acid 0·88 mg ml^{-1}.

Incubations

Triplicate soil samples (equivalent to 25 g oven-dry weight) were amended with 2·1 ml of either: (a) raw undiluted effluent; (b) effluent diluted 1:1; (c) effluent diluted 1:2; or (d) water only. Amendments represented effluent applications rates of 5 (high), 2·5 (medium), 1·7 (low, recommended) and 0 (zero) l m^{-2}, and were calculated assuming a soil bulk density of 1·19 g cm^{-3} and a soil depth of 5 cm (1 m^2=59500 g dry soil). Silage effluent was applied to the surface of the field-moist soil (21% moisture content) and was then thoroughly mixed in. Following amendment with silage effluent, soil moisture content was 42% (oven-dry weight basis). Amended soils contained in beakers were incubated individually in sealed 1·8 litre Agee jars; each contained two vials, one with 10 ml 2 M NaOH to absorb evolved CO_2, and one with 10 ml CO_2-free water to maintain humidity and avoid any loss of soil moisture during the incubation period. The soils were incubated at 25°C for up to 32 days. Periodically (on days 2, 4, 8, 16 and 32), triplicate jars were taken and beakers of soil and vials of NaOH were removed for analysis of microbial characteristics. Soil pH measured in H_2O (1:2·5 w/v), as described by Blakemore *et al.* (1987).

An additional experiment was set up to examine the impact of increasing application rates of silage effluent, far greater than those used above, on microbial activity. In this experiment, soils were amended with 7·5 ml (adjusting soil moisture content to 84%) of increasing concentrations of silage effluent as follows: 0, 5, 10, 15, 20, 25 and 30 l m^{-2}. Different concentrations of silage effluent were made up by diluting with appropriate quantities of H_2O. Amended soils were incubated with a CO_2 trap (2 M NaOH) as above at 25°C for 3 days and removed for measurement of CO_2 respiration and dehydrogenase activity only.

All data were analyzed by analysis of variance and Student's *t*-test (GENSTAT). Data from the second experiment were analyzed by regression analysis.

CO_2 respiration

The total CO_2 absorbed in NaOH was measured by titration of aliquots (0·1 ml) of alkali against 0·1 M HCl with phenolphthalein as indicator (Hesse, 1971), to determine the residual alkali, after first precipitating out carbonates by addition of 25 ml 10% $BaCl_2$. Rates of CO_2 evolution were expressed as μg CO_2-C g^{-1} dry soil.

Biomass C

The microbial biomass was determined by the fumigation–extraction method (Vance *et al.*, 1987). Fumigated ($CHCl_3$ for 24 h at 25°C) and non-fumigated soils (5 g dry weight equivalent) were extracted with 0·5 M K_2SO_4 for 30 min (1:5 soil to extract ratio) and filtered and then an aliquot (8 ml) was analyzed for organic C by the acid–dichromate oxidation method (Tate *et al.*, 1988). Additional oxidizable C obtained from fumigated soil was taken to represent the microbial-C flush and converted to microbial biomass C using the relationship: microbial C=C flush/0·35 (Sparling *et al.*, 1990). Microbial biomass expressed as μg biomass-C g^{-1} dry soil.

Enzyme activities

Dehydrogenase activity was measured as the production of formazan from iodonitrotetrazolium chloride using the methodology of von Mersi and Schinner (1991). Urease activity was measured using the methodology of Nannipieri *et al.* (1978), except that 6% urea was added to soil samples (1 g fresh

weight). Phosphatase activity was measured using the methodology of Tabatabai and Bremmer (1969) with modifications suggested by Speir *et al.* (1984).

RESULTS AND DISCUSSION

Soil pH

In all soils, the pH ranged from 5·9 to 6·6 over the incubation period and the application of different rates of silage effluent had no significant effect (data not shown).

Microbial biomass

In the three amended soils and the control, the amount of microbial biomass C was greatest after 4 days of incubation and thereafter declined significantly ($P<0·001$) over the incubation period of 28 days [Fig. 1(a)]. At days 2 and 4 of the incubation period, microbial biomass C was significantly ($P<0·001$ and $P<0·01$, respectively) different in all four soils, and was consistently greater in soils receiving increasingly higher rates of silage effluent application. However, after 8 and 16 days of incubation, microbial biomass C was not significantly different in the four soils, although at these sample dates biomass C was significantly ($P<0·05$) greater on the soil amended with 5 l m^2 (high) than in the control. When microbial biomass C was at a maximum (day 4), values ranged from 970 μg biomass C g^{-1} in the control soil to 2257 μg biomass C g^{-1} in the soil receiving an equivalent of 5 l m^{-2} silage effluent. At the start of the experiment the unamended soil had a microbial biomass of 820 μg C g^{-1} soil, therefore the addition of silage effluent at a rate of 5 l m^{-2} resulted in an almost three-fold increase in microbial biomass. The application of water alone (control) resulted in an 18% increase in microbial biomass after 4 days. These results suggest that the application of silage effluent (up to 5 l m^{-2}) to a common grassland soil has no detrimental effect on microbial biomass and, in fact, produces a short-term increase in the abundance of microorganisms.

As in other studies of soils amended with manures and crop residues (Martens *et al.*, 1992; Wardle, 1992; Kandeler & Eder, 1993), the increase in microbial biomass is probably related in part to the input of readily-available nutrient substrates into the soil. Indeed, the addition of silage effluent to soil at an equivalent rate of 5 l m^{-2} provided approximately 800 μg of soluble carbohydrate g^{-1} soil for utilization by the native microbial biomass. Assuming all available carbohydrate was utilized for biosynthesis, this suggests that approximately 60% of the increase in microbial biomass in the heavily-amended soil (5 l m^{-2} silage effluent) may be due to the input of available carbon derived from silage effluent.

The above finding suggests that there was also a considerable input (approximately 40%) of microorganisms into the soil from the silage effluent, including lactic acid bacteria, enterobacteria and yeasts, which can all be found in high numbers in silage effluent (R. J. Merry, personal communication). This may also explain in part the rapid fall in microbial biomass observed at days 8 and 16 in amended soils, since the majority of bacteria present in silage effluent are facultative anaerobes which may be able to survive in an aerobic soil environment for only a short period of time.

Rapid increases in soil microbial biomass, following the application of silage effluent to grassland, are likely to result in temporal biological immobilization of effluent-derived N and P. Such immobilization, and the subsequent slow release of nutrients by the biomass, may have a role in reducing the risk of leaching of unacceptable levels of nutrient into adjacent drainage systems.

CO_2 evolution

Accumulated CO_2 evolution was significantly ($P<0·001$) different in all four soils throughout the incubation period, being greatest in the soils receiving high rates of silage effluent application (5 l m^{-2})

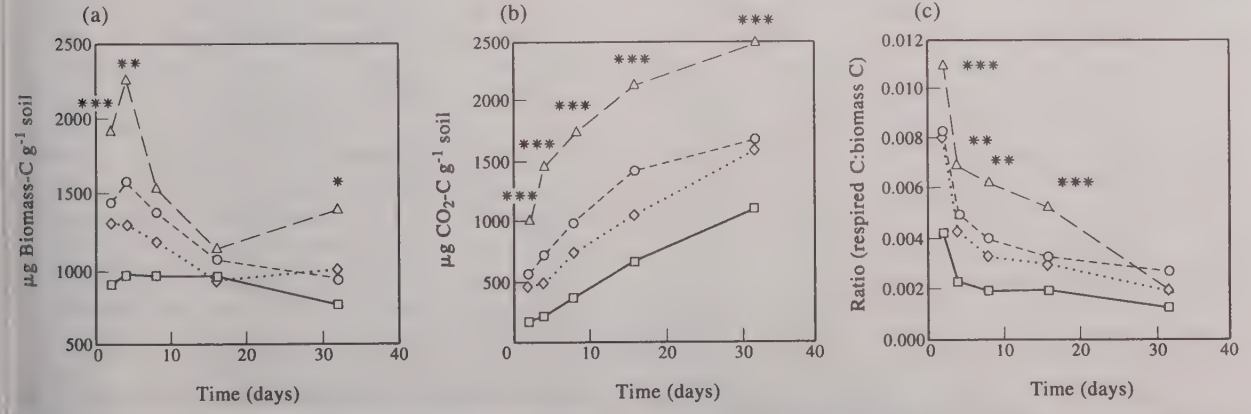

Fig. 1. Effect of silage effluent application rate (l m^2) on (a) microbial biomass C (b) accumulated CO_2 evolution and c) the ratio. Application rates are shown as —□— unamended control, ..◇.. low (1·7 l m^2), --○-- medium (2·5 l m^2) and --△-- high (5 l m^2); *, **, *** represent $P<0·05$, $P<0·01$, $P<0·001$, respectively.

and least in the unamended control soil [Fig. 1(b)]. Similarly, in the second experiment, CO_2 evolution increased linearly ($r^2=0{\cdot}98$; $P<0{\cdot}001$) with increasing rates of silage effluent application up to an equivalent of 30 l m^{-2} (Fig. 2). This finding was despite the fact that moisture contents (84%) in the amended soils were above optimal for microbial activity (approximately 50–60% moisture). However, these high moisture conditions may, in fact, be more realistic of those experienced in the field immediately following the application of diluted silage effluent to typically wet and heavy textured grassland soils of the western UK.

In the first experiment, there was a consistent trend of increasing CO_2 evolution with increasing rates of silage effluent application [Fig. 1(b)]. At the end of the incubation period accumulated CO_2 evolution ranged from 1108 μg CO_2-C g^{-1} in the unamended control soil to 2438 μg CO_2-C g^{-1} in the soil receiving an equivalent of 5 l m^{-2} silage effluent. The maximum rate of CO_2 evolution in all three amended soils was at day 2 and rates progressively declined after this sample date. These findings are in agreement with those of Cooper (1977) and Jones (1986) who also showed that the application of silage effluent to soils has no apparent detrimental effect on microbial activity, measured as CO_2 evolution, and results in a temporal increase in microbial respiration. As with microbial biomass, this temporal increase in microbial activity is likely to be related to the input of readily-available substrates into the soil and also to the presence of microorganisms derived from the silage effluent which are capable of utilizing volatile fatty acids, such as lactic acid.

Despite the above findings, specific respiration (i.e. ratio respired C:biomass C) was also shown to increase at the higher rates of silage-effluent application, throughout the incubation period [Fig. 1(c)]. This may suggest that although microbial biomass and respiration were higher in soils amended with silage effluent, the microorganisms were physiologically stressed, resulting in a relative increase in the diversion of C from biosynthesis of new biomass to maintenance requirements (respiration). Such physiological stress may be related in part to the presence of microbial toxins, such as volatile fatty acids, or to increased competition for available substrates to the native microbial biomass. This finding, however, is contrary to those of other studies, which have shown that the application of easily available substrates to soils, via cattle slurry (Kandeler & Eder, 1993) and inorganic fertilizers (Insam *et al.*, 1991), results in a decrease in the specific activity of microorganisms, since C is no longer limiting.

Increases in specific respiration may also be an indication of shifts in microbial community structure in the amended soils. It is well known that bacteria have a higher metabolic activity than fungi (Anderson & Domsch, 1975) and that silage effluents, particularly during the early stages of silage fermentation, have a predominantly bacterial population (R. J. Merry, personal communication). Thus, it is possible that the increased specific respiration in soils amended with silage effluent may be due to an increase in the proportion of bacteria relative to fungi. This shift may be a consequence of the addition of effluent-borne bacteria to the soil, or alternatively an increase in the competitive ability of native soil bacteria, relative to fungi, in response to added substrates.

Enzyme activities

The application of different rates of silage effluent to soils had no consistent effect on the activities of both phosphatase and urease throughout the incubation period [Fig. 3(a) and (b) respectively]. However, dehydrogenase activity (an index of total aerobic microbial activity) was significantly ($P<0{\cdot}01$) affected by the treatments at days 2 and 4, being greatest in the soil receiving the equivalent of 5 l m^{-2} silage effluent and least in the unamended control [Fig. 3(c)]. After day 4, dehydrogenase activity declined progressively until there were no differences between the soils at day 8 and thereafter. In the second experiment, dehydrogenase activity increased

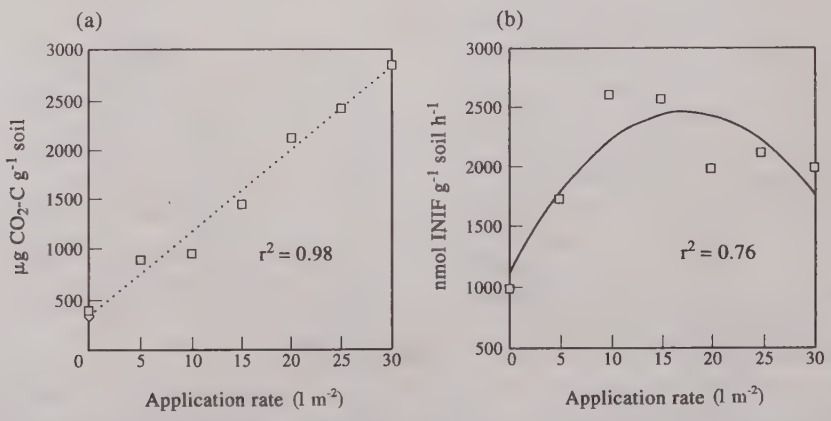

Fig. 2. Relationship between (a) silage effluent application rate and CO_2 evolution and (b) dehydrogenase activity.

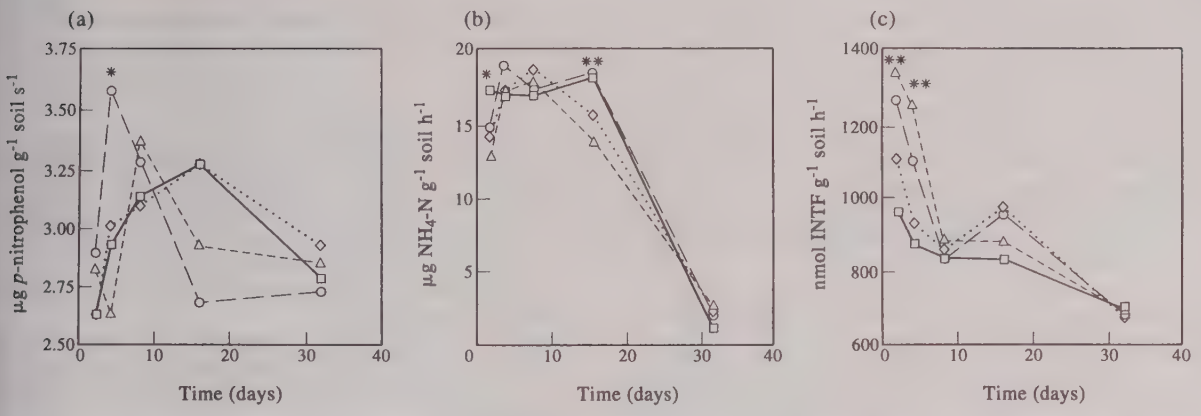

Fig. 3. Effect of silage effluent application rate (l m^2) on (a) phosphatase activity, (b) urease activity and (c) dehydrogenase activity. Application rates are shown as —□— unamended control, ..◇.. low (1·7 l m^{-2}), --○-- medium (2·5 l m^{-2}) and --△-- high (5 l m^{-2}); *, **, represent $P<0·05$, $P<0·01$, respectively.

up to an application rate equivalent to 15 l m^{-2} and then appeared to decline steadily above this level. The relationship was described as polynomial ($r^2=0·76$) [Fig. 2(a)]. It is possible that dehydrogenase activity is a more sensitive indicator of microbial activity than CO_2 evolution, suggesting that beyond an application rate equivalent to 15 l m^{-2} microbial activity is inhibited. However, it is also possible that at these unrealistically high rates of silage-effluent application (i.e. >15 l m^{-2}) an abiological factor, such as high concentration of specific chemical compounds, is adversely affecting the enzyme assay. This suggestion would be in line with other recent studies of soils amended with sewage-sludge (e.g. Chander & Brookes, 1991), which have called into question the use of the dehydrogenase assay in studies of polluted soils.

CONCLUSION

The present laboratory study suggests that the application of silage effluent to a grassland soil results in a short-term increase in both the abundance and activity of microorganisms. There appears to be no short- or long-term detrimental effect of applying high rates of silage effluent to soil on microbial biomass and activity. However, there is an indication that the higher microbial biomass in amended soils may be temporarily physiologically stressed (i.e. high metabolic quotient) and/or there are shifts in the structure of the microbial community of amended soil in favour of effluent-derived bacteria. It is possible that long-term detrimental effects of high application rates of silage effluent were masked by the increased aeration of sieved soils, as used in this laboratory study. Further studies in the field and/or the use of intact cores are required to examine the spatial impacts of silage effluent in soils, particularly in relation to soil structure and the creation of temporal anaerobic conditions within isolated soil pores and fissures of poorly-drained soils. There is also a need for further studies to examine the potential toxic effect of different chemical compounds within silage effluent, which are known to vary greatly during the ensiling process (Jones *et al.*, 1990).

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SAUSSUREA LAPPA (KUTH) AS A NEW SOURCE OF INULIN FOR FERMENTATIVE PRODUCTION OF INULINASE IN A LABORATORY STIRRED FERMENTER

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Abstract

Inulinase was produced by Aspergillus niger *van Teighem UV11 in a 10 l glass fermenter using readily-available nitrogenous by-products, corn steep liquor (CSL), defatted groundnut meal and defatted soya-bean meal, in different concentrations with kuth (*Saussurea lappa*) root powder as a source of inulin (as inducer), under varying agitation conditions. With the combination of kuth (1·0%), aeration (1·5 vvm) and agitation (300 rpm), the maximum yield of insulinase obtained was 290 Uml^{-1} after 72 h.*

Key words: Aspergillus niger, fermenter, inulinase, *Saussurea lappa*.

INTRODUCTION

Inulin is a polymer of plant origin that serves as a reserve carbohydrate in Compositae and Graminae. Jerusalem artichoke and chicory plant roots containing inulin have been used for fermentative production of inulinase by microorganisms (Manzoni & Cavazzoni, 1992; Gupta *et al.*, 1988). *Saussurea appa* (kuth or costus — common name) belongs to the Compositae family and contains inulin in the roots. Kuth root as a rich source of inulin has been utilized for high-fructose-syrup production by chemical hydrolysis (Kulkarni *et al.*, 1969). Kuth root powder as a source of inulin for inulinase fermentation has not been reported. In the present studies, kuth root powder was used as the source of inulin for inulinase production in a 10 l fermenter.

METHODS

Carbon and nitrogen analyses

Total carbon and nitrogen contents of the kuth root powder [local market, Bombay, India and powdered in a flour mill to a very fine powder (60 mesh)] and corn steep liquor (CSL; Anil Starch Ltd, Ahmedabad) were determined by Total Carbon Analyzer (Beckmann, USA) and Kjeldhal methods respectively.

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Inulin content

Inulin content of kuth root powder was determined using Streptakov phosphomolybdic-permanganate (Winton & Winton, 1960).

Preparation of inoculum

Aspergillus niger van Teighem UV11, the UV mutant of a soil isolate (Poorna & Kulkarni, 1994), was subcultured on the synthetic medium agar of Allais *et al.* (1987) and incubated at 30±2°C for 72 h. The spores were then harvested and suspended in 0·85% saline containing 0·01% Tween 80 to obtain $2{\cdot}5 \times 10^6$ spores per ml (by haemocytometer count). A 5% (v/v) inoculum of this suspension was used.

Medium

The medium (B) used for fermentation had the following composition (per litre): kuth root powder, 10 g; CSL, 10 g; KH_2PO_4, 5 g; trace elements solution, 1 ml; silicone oil, 0·001%; and pH 5·4. A total volume of 6 l was used for every batch of fermentation studies. The medium was sterilized by autoclaving at 121·6°C for 15 min. Aeration was maintained at 1·5 vvm and agitation at 225 rpm unless otherwise stated; 20 ml samples were withdrawn at specific intervals to assay for inulinase activity.

The composition of the medium and volume of inoculum used was the same for all parameters studied unless otherwise stated. Effect of different process parameters was studied as follows. Fermentations were carried out in duplicate and average results are given.

Time course analysis of inulinase fermentation

About 20 ml samples were siphoned out of the 10 l glass fermenter (Enmvee Co. Ltd, Pune) every 24 h and assayed for inulinase activity, reducing sugar content of the medium and pH.

Effect of aeration
The medium in the fermenter was aerated at 1, 1·5, 2 and 3 vvm at the agitation rate of 225 rpm. Inulinase activity was estimated at the end of 72 h.

Effect of agitation
At constant aeration of 1·5 vvm, agitation rates of 115, 225, 300 and 400 rpm were maintained. Inulinase activity was estimated at the end of 72 h.

Effect of nitrogen sources
Different complex nitrogen sources, CSL, defatted soya-bean flour and defatted groundnut flour (Godrej Soaps Ltd, Bombay, India) were used at 1% concentration separately in the medium (B) described earlier. The aeration rate was maintained at 1·5 vvm and agitation at 300 rpm. Inulinase activity and biomass were determined at the end of 72 h.

Determination of biomass
The mycelial mass of *A. niger* van Teighem UV11 was collected by centrifugation of the culture medium at 10000 rpm for 25 min. The biomass was determined after washing the mycelial mass with distilled water and drying in an oven at 80°C to constant weight.

Inulinase assay
To 2 ml of 0·2% inulin (Sigma) and 2 ml of 0·01 M acetate buffer (pH 4·6) was added 0·5 ml of appropriately diluted enzyme preparation (culture filtrate) and incubated at 50°C for 20 min. After incubation, the tubes were kept in a boiling water bath for 10 min to inactivate the enzyme. The centrifuged reaction mixture was assayed for reducing sugar, in terms of fructose formed, by the DNSA method (Miller, 1959) using fructose as standard. One unit of inulinase (U) was defined as the amount of enzyme which produced 1 μmole of fructose under the assay conditions as described above.

Table 1. Analysis of kuth root powder and CSL

Analysis (%)	Kuth root	CSL
Inulin	30·0±1·0	—
Total nitrogen	0·34±0·05	4·32±1
Total carbon	45·06±1·0	22·70±1

RESULTS AND DISCUSSION

Table 1 shows the total carbon and nitrogen content of kuth root powder and CSL. In the kuth root powder the inulin content was lower than that of Jerusalem artichoke (58%) or Dhalia (44%) (Doby, 1965). Extraction of inulin from the roots involves diffusion with steam, which is repeated twice for complete extraction. These steps require input of energy which makes fermentation more costly. Manzoni and Cavazzoni (1992) reported that the temperature and pressure involved during autoclaving are needed to extract inulin from Jerusalem artichoke root powders. Hence, in all the experiments in the present studies, kuth root powder was directly used without any prior extraction as a source of inulin.

After the preliminary understanding of media and process parameters affecting inulinase production in shake flask cultures, fermentation was attempted in a 10 l glass fermenter. The time course analysis of inulinase fermentation in the fermenter is shown in Fig. 1. The reducing sugar content in the medium increased with time reaching a maximum of 500 $\mu g ml^{-1}$ in 96 h. The highest inulinase activity of 280 $U ml^{-1}$ was obtained in 72 h at the aeration rate of 1·5 vvm and agitation at 225 rpm. The pH shifted from the initial 5·4 to 6·0 at 120 h. One point five

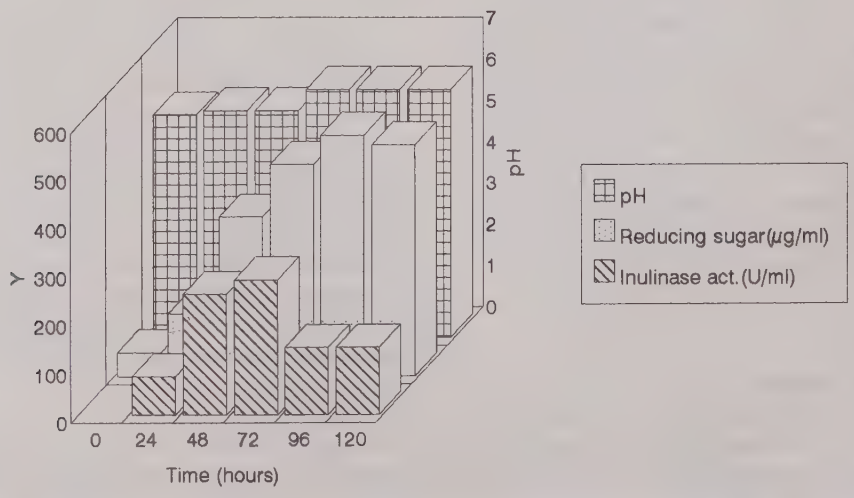

Fig. 1. Time course of inulinase production in a stirred fermenter. Six litres medium in a lab-stirred 10 l fermenter incubated at 30°C with aeration at 1·5 vvm, agitation 225 rpm and inoculum level at 5%, for 120 h. Y — Units of inulinase activity (U/ml) and reducing sugar content (μg/ml) in the medium.

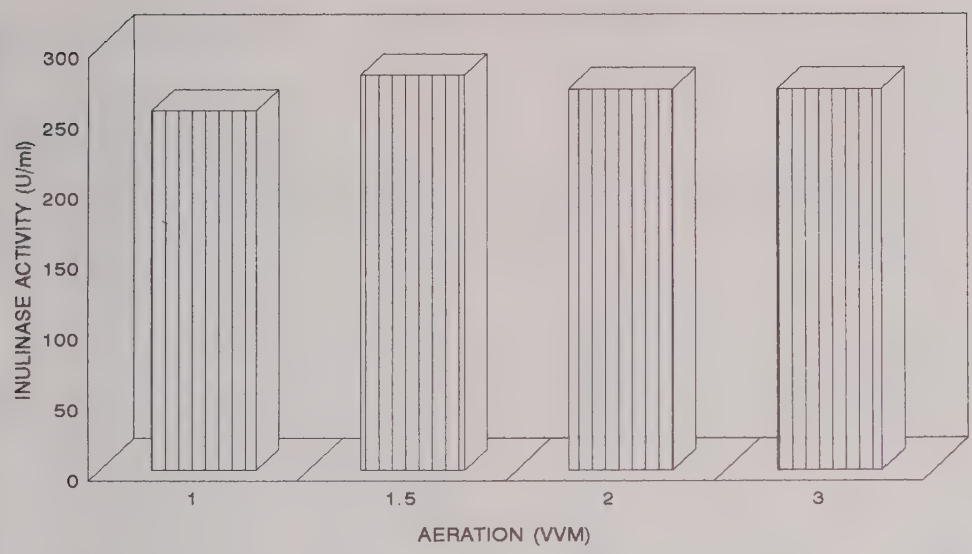

Fig. 2. Effect of aeration on inulinase production. Six litres medium in a lab-stirred fermenter inoculated with 5% inoculum and incubated at 30°C, agitation at 225 rpm and at varying aeration rates. Inulinase assay was carried out at the end of 72 h.

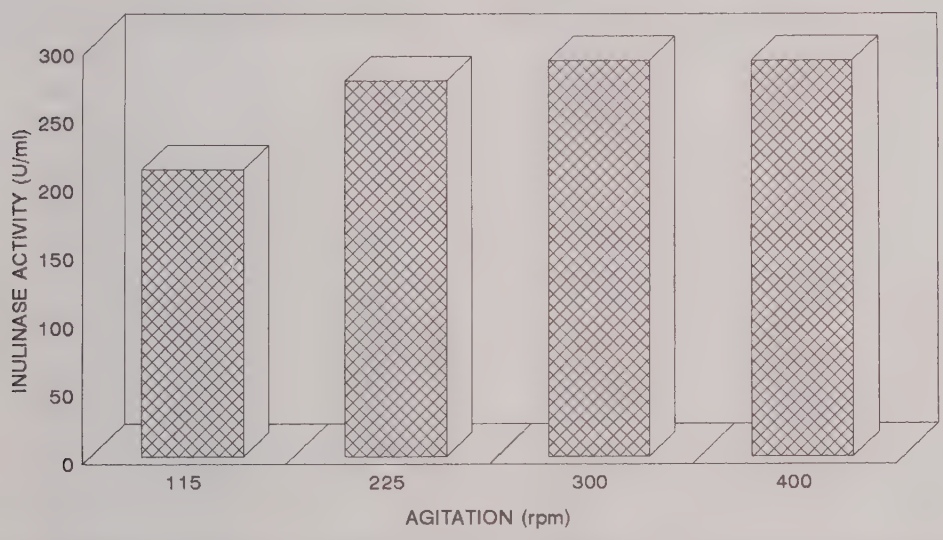

Fig. 3. Effect of agitation on inulinase production. Six litres medium in a lab-stirred fermenter inoculated with 5% inoculum and incubated at 30°C with aeration at 1·5 vvm and at varying agitation rates. Inulinase assay was carried out at the end of 72 h.

Table 2. Effect of varying nitrogen sources on inulinase production in a fermenter[a]

Nitrogen source (1%)	Inulinase activity (μml^{-1})	pH	Biomass dry wt (g per 6 l)
CSL	290	5·5	36·0
Defatted soya-bean meal	160	6·0	18·0
Defatted groundnut meal	135	6·0	13·0

[a]Six litre medium (B) with different nitrogen sources at 1% concentration was inoculated with 5% inoculum and incubated at 30±2°C for 72 h with aeration at 1·5 vvm and agitation at 300 rpm.

vvm aeration at an agitation of 225 rpm gave the highest inulinase activity of 280 Uml^{-1} (Fig. 2). There was no effect on inulinase production of increased aeration. However, increasing the agitation rate to 300 rpm with 1·5 vvm aeration gave an increased inulinase yield of 290 Uml^{-1} (Fig. 3). Thus, with the aeration at 1·5 vvm and agitation at 300 rpm fermentation of kuth by *A. niger* van Teighem UV11 for inulinase production was tested with various readily available nitrogenous by-products at 1% concentration (Table 2). CSL gave the highest yield.

Based on the results obtained in the fermenter,

the composition of the medium and aeration–agitation conditions for inulinase production can be summarized as follows: kuth root powder, 1%; CSL, 1%; pH 5·4. Using this medium, with time of incubation 72 h, aeration 1·5 vvm and agitation 300 rpm, the inulinase yield could be 290 Uml^{-1}. Fermenter-based inulinase production using pure commercially available inulin has been reported for *K. fragilis* (100 Uml^{-1}) (Grootwassink & Fleming, 1980) and *K. marxianus* (212 Uml^{-1}) (Parekh & Margaritis, 1985). Unfortunately, there are no reports available on production of inulinase in a fermenter with any of the identified fungal inulinase producers. Also, there are no reports on use of kuth root as a source of inulin for inulinase production. Hence it is difficult to compare the present results with any of the published data. The present work has, therefore, identified for the first time the possibility of using *A. niger* van Teighem and also kuth root for inulinase production.

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EMISSIONS AND POWER CHARACTERISTICS OF DIESEL ENGINES ON METHYL SOYATE AND DIESEL FUEL BLENDS*

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Abstract

A Detroit 6V-92 TA coach diesel engine and a Cummins NTA-855-C diesel engine were operated on fuels produced by blending methyl soyate with No. 2 diesel fuel. Seven fuels, namely, neat diesel fuel; 90:10, 80:20, 70:30, 50:50 and 25:75% (v/v) blends of diesel fuel:methyl soyate; and neat methyl soyate were prepared and tested for the performance of the Detroit engine using standard test code SAE J1349. Specific gravity, viscosity, and heating values of all seven fuels were determined. Power developed by the engine, brake specific fuel consumption, and emissions analyses including brake specific hydrocarbon, brake specific oxides of nitrogen, brake specific carbon monoxide and smoke were measured. The performance of the engine was found to be satisfactory without a significant reduction in power output on methyl soyate blends up to 30%. Based on these results, the Cummins engine was run on four fuel blends, namely, neat diesel fuel; and 90:10, 80:20 and 70:30% (v/v) blends of diesel:methyl soyate. The performance of the Cummins engine was found to be satisfactory without any significant reduction in power. Engine exhaust emissions, except NO_x, were reduced for both engines as the concentration of the methyl soyate in the fuel increased.

Key words: Methyl soyate, biodiesel, Detroit engine, Cummins engine, emissions, power, hydrocarbon, nitrous oxides, smoke.

INTRODUCTION

Interest in the development of alternative fuels has grown steadily during the past two decades. Fear of dependence on foreign-controlled fuel sources of finite supply, prompted by oil shortages and rising prices initiated research in the areas of renewable alternate fuels. Among the different renewable fuels considered for use were various types of oils available from plant and animal sources. These oils have potential as diesel fuels but there is a need for a continuous and concentrated research effort (Quick, 1980). From the environmental point of view also, diesel engines are a major source of air pollution (Glassman, 1987). The exhaust gases from diesel engines contain oxides of nitrogen (NO_x), carbon monoxide (CO), organic compounds consisting of unburnt or partially burned hydrocarbons (HC), and particulate matter (consisting primarily of soot). The petroleum industries are presently hard-pressed to use and produce a more environmentally friendly fuel. Specifically, they have been expected to lower the sulfur content and particulate emissions.

Renewable fuels derived from vegetable oils are capable of providing good engine performance in the short term (Goering & Fry, 1984). In more extended operations, the same fuels have caused degradation of engine performance, excessive carbon and lacquer deposits and actual damage to the engine. The probability of such problems occurring is influenced by the type and physical properties of the fuel. These oils are too viscous for prolonged use in direct-injected diesel engines (Ziejewski *et al.*, 1984). The high viscosity of vegetable oils leads to poor fuel atomization and inefficient mixing with air, which contributes to incomplete combustion (Bagby, 1987). Also, because of their unsaturation, vegetable oils are inherently more reactive then diesel fuels; they are more susceptible to oxidative and thermal polymerization reactions, which can interfere with combustion (Korus *et al.*, 1982). Because of their high molecular weights (about 880), vegetable oils have low volatility. These chemical and physical properties cause vegetable oils to accumulate and remain as charred deposits when they contact engine cylinder walls.

The problem of charring and deposition of oils on the injector and cylinder wall can be overcome by reducing the viscosity of the vegetable oils. Esterification of oils is a process by which glycerol is

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removed from the oil and esters, with a lower viscosity, are obtained. The esters of vegetable oils have fuel properties that compare much better with diesel fuel than do neat vegetable oils (Wagner *et al.*, 1984).

The purpose of this investigation was to determine the effects of fueling a diesel engine with methyl soyate (methyl ester of soybean oil) and No. 2 diesel fuel blends on power characteristics and emissions analyses. The specific objectives were (1) to evaluate the performance of diesel engines using blends of methyl soyate and No. 2 diesel fuel and (2) to conduct emission quality evaluations on the same blends.

METHODS

Experimental engines and instruments

A Detroit Diesel 6V-92 TA Coach, 206 kW (277 hp) diesel engine and a Cummins NTA-855-C Big Cam III, 298 kW (400 hp) diesel engine were used in this study. Specifications of both engines are presented in Table 1.

The engines were coupled to an Eaton 522 kW (700 hp) dynamatic eddy current dry gap dynamometer (EATON Power Transmission Systems, Eaton Corp., Kenosha, WI) with a DANA 1810 u-joint coupler. Engine torque was measured with a load cell and a Daytronic integrator (9000 Modular Instrument System, Daytronic Corp., Miamisburg, OH), and speed was measured using a 60-tooth wheel and a magnetic pick-up attached to the dynamometer. Fuel consumption was measured with an EM Corp. (Lincoln, NE) custom-built mass measurement system in which fuel weight was measured over an operator-selected time period. Air flow into the engine was measured with a Badger BVT-IF venturi flow meter (Badger Meter, Inc., Tulsa, OK). The air flow meter was positioned in a 0·15 m diameter, 5·2 m long pipe with a surge tank between the meter and the engine. A throttle valve was used at the inlet of the surge tank to control engine inlet pressure. The exhaust system consisted of 2·1 m of 0·13 m diameter exhaust tubing leading into a 0·25 m diameter duct to the outside air. A centrifugal fan provided exhaust ventilation. A throttle valve was positioned in the exhaust tubing to control exhaust back pressure.

Temperatures were measured using type K and type T thermocouples and a EDTCS 100 Horiba dynamometer test stand system (Omega Engineering, Inc., Stamford, CT). Pressures were measured with analog gages and manometers (H_2O and Hg) calibrated with a dead-weight tester.

Oxides of nitrogen (NO/NO_x) were measured with a Beckman model 955 chemiluminescent analyzer (Beckman Industrial Corp., La Habra, CA). Hydrocarbons (HC) were determined by a total HC analyzer model JUM VE7 flame ionization detector, (J.U.M. Engineering, Karlsfeld, Germany). This analyzer was designed to continuously measure the concentration of total organic HC in gaseous samples. Carbon monoxide (CO) was measured with a Beckman non-dispersive infrared analyzer model 880-A (Rosemount Analytical, Inc., La Habra, CA). Smoke units were measured with a Bosch EFAW 65-A smoke evaluation instrument (Robert Bosch GMBH, Stuttgart, Germany).

Fuels

The following test fuels were used in this study:

1. 100% No. 2 diesel fuel (baseline);
2. 90:10% (v/v) diesel:methyl soyate blend;
3. 80:20% (v/v) diesel:methyl soyate blend;
4. 70:30% (v/v) diesel:methyl soyate blend;
5. 50:50% (v/v) diesel:methyl soyate blend;
6. 25:75% (v/v) diesel:methyl soyate blend;
7. 100% methyl soyate.

The Detroit diesel engine was run on all seven fuel blends, while, on the basis of the performance of this engine, a cut-off point for diesel:methyl soyate blend was decided to be 70:30% (v/v) and thus a Cummins engine was run on four fuel blends of 100% diesel fuel, 90:10, 80:20 and 70:30% (v/v) blends of diesel:methyl soyate.

The diesel fuel used in this study was a high sulfur (0·24%)-containing fuel. Methyl soyate was procured from Interchem, Inc. (Kansas City, MO). Density, viscosity and energy content of methyl soyate were determined. The density of methyl soyate was determined by standard ASTM hydrometer method D

Table 1. Engine specifications

Specifications	Detroit 6V-92 TA Coach engine	Cummins NTA-855-C engine
Type of engine	6 cylinder, 2-stroke, direct injection	6 cylinder, 2-stroke, direct injection
Power (rated)	206 kW (277 hp)	298 kW (400 hp)
Bore × stroke	123 mm × 127 mm	140 mm × 152 mm
Displacement	9·05 l	14 l
Compression ratio	17·0	14·0
Valves per cylinders	4	4
Aspiration	Turbocharger and blower assisted	Turbocharged
Turbocharger	Garret TV 7512	Holsett type BHT 3B
Cooling system	Jacketed water aftercooler	Jacketed water intercooler

1298-85 (ASTM, 1993). Viscosity was determined with a Brookfield Synchro-Lectric LV viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, MA) with a UL adapter at 40°C. The temperature of the sample was maintained within ±0·5°C with a constant temperature bath (Tamson, Neslab Instruments, Inc., Portsmouth, NH). Five viscosity measurements were made on each sample and an average was taken. The energy contents of methyl soyate and its blends were determined by standard test method ASTM D 240-87 (ASTM, 1991) for heat of combustion of liquid hydrogen fuels by bomb calorimetry.

Test run and performance map

Engine testing on the above fuels was performed using standard test code SAE J1349 (SAE, 1993*b*). The testing was done in the Nebraska Power Laboratory at the University of Nebraska-Lincoln. The sequence of fuels used was in the order given above. With each fuel blend, standard performance and exhaust emission data were recorded and the engine was operated at the following operating points using code SAE J1312 (SAE, 1993*a*):

Engine speed for Detroit diesel engine, rpm	Engine speed for Cummins diesel engine, rpm	Torque, %
2100	2100	100
1950	2000	100
1800	1800	100
1650	1600	100
1500	1400	100
1350	1300	100
1200	1200	100
1050	—	100

Testing procedure

The engine was warmed-up at low idle long enough to establish correct oil pressure and was checked for any fuel, oil, water and air leaks. The speed was then increased to 1600 rpm and sufficient load was applied to raise the coolant temperature to 71°C (160°F). After completion of the warm-up procedure, the intake and exhaust restrictions were fixed at rated engine speed and full power and from then on were not adjusted for different speeds or load changes after initial settings were completed.

The engines were run at the speeds specified above on the different fuel blends given above at full load for at least 6 min and data were recorded during the last 2 min of operation at each speed. The response variables included power, torque, HC, CO, NO_x and smoke. These data were recorded at 5 s intervals for 2 min using an AutoNet computer program and averaged over that period.

Experimental design and data analysis

Detroit diesel engine

Treatments were arranged as a full two-factor factorial with a single experimental unit per treatment combination. The two factors considered were engine speed at eight levels and diesel fuel:methyl soyate blends at seven levels. An experimental unit was the 6 min of operation under a treatment combination. Statistical analyses (SAS, 1989) were conducted in three steps using analysis of variance (ANOVA) and regression.

First, an ANOVA model was fit on the full factorial with the two-way interactions considered as an acceptable estimate of experimental error. Each main effect and the interactions were broken into one degree of freedom orthogonal polynomial contrasts. In the second step, a new ANOVA model was fit with all non-significant ($P > 0{\cdot}005$) two-way interactions from the first step pooled with error. All remaining terms were tested for significance against the pooled error. In the third step, all significant terms identified in the second step were fit in a regression model. The estimated model was then used to generate predicted points on a response surface, which was then plotted.

Cummins engine

Treatments were arranged as a full three-factor factorial with a single experimental unit per treatment combination. The three factors considered were baseline at two levels (first and second), engine speed at seven levels and diesel fuel:methyl soyate blends at four levels. An experimental unit was the 6 min of operation under a treatment combination. Statistical analyses (SAS, 1989) were conducted in three steps using analysis of variance (ANOVA) and regression.

First, an ANOVA model was fit on the fuel factorial with the 3-way interactions considered as an acceptable estimate of experimental error. Each main effect and the interactions, except the three-way, were broken into one degree of freedom orthogonal polynomial contrasts. In the second step, a new ANOVA model was fit with all non-significant ($P > 0{\cdot}005$) two-way interactions from the first step pooled with error. All remaining terms were tested for significance against the pooled error. In the third step, all significant terms identified in the second step were fit in a regression model. The estimated model was then used to generate predicted points on a response surface, which was then plotted.

RESULTS AND DISCUSSION

Fuel properties

In order to evaluate the potential of the methyl soyate used in this study, physical properties, including density, viscosity and heating values, were evaluated. Table 2 summarizes these properties of methyl soyate, with and without diesel fuel.

The specific gravities of the methyl soyate and blends were found to be greater than that of the diesel fuel, thus giving lower corresponding API gravities. The specific gravity of methyl soyate

Table 2. Physical properties of methyl soyate and its blends with No. 2 diesel fuel

Blends	Specific gravity at 15·6/15·6°C (60/60°F)	API gravity	Viscosity at 40°C (mPa-s)	Heating value	
				Mass basis (MJ/kg)	Volume basis at 15·6°C (MJ/l)
Methyl soyate (100%)	0·8870	28·03	4·06	40·07	35·55
Diesel fuel + methyl soyate (25:75% v/v)	0·8735	30·49	3·51	40·98	35·80
Diesel fuel + methyl soyate (50:50% v/v)	0·8590	33·23	3·05	41·95	36·04
Diesel fuel + methyl soyate (70:30% v/v)	0·8445	36·05	2·58	42·93	36·26
Diesel fuel + methyl soyate (80:20% v/v)	0·8430	36·35	2·41	44·31	37·35
Diesel fuel + methyl soyate (90:10% v/v)	0·8415	36·65	2·28	44·78	37·84
Diesel fuel (100%)	0·8383	37·29	2·07	45·50	38·14

(0·887) was maximum and values decreased with increasing diesel fuel content.

The API gravities increased with increasing diesel fuel contents. The API gravity of the methyl soyate was less than the API gravity of the diesel fuel. This was due to the fact that API gravity depends on specific gravity and as the specific gravity of the methyl soyate was large compared to diesel fuel, the API gravity was less. The API gravity of a distillate fuel indicates the following general relationships (Wagner *et al.*, 1984). (1) The lower the API gravity, the more viscous the fuel and the higher the carbon residues. (2) The higher the API gravity (less mass per unit volume of fuel), the lower the heating value per unit volume, but the greater the heating value on a mass basis. (3) Higher API gravity implies higher cetane number.

The viscosity of the methyl soyate was observed to be twice as high as the viscosity of diesel fuel at 40°C. The viscosity of methyl soyate was 4·06 mPa-s, whereas the viscosity of diesel fuel at 40°C was 2·07 mPa-s. Thus, the statement relating low API gravity with high viscosity was correct. The viscosities of the methyl soyate and diesel fuel blends decreased with increasing diesel fuel content.

The heating value of the methyl soyate was 11·9% lower, on a mass basis, than the reference diesel fuel, as presented in Table 2. The heating values of the blends increased with increasing diesel fuel content. The methyl soyate and its blends had higher specific gravities than diesel fuel, so that the energy content of the methyl soyate, on a volume basis, was somewhat closer to diesel fuel than on a mass basis. The energy content of methyl soyate was only 6·7% lower than diesel fuel, on a volume basis. Since the injection pump meters fuel on a volume basis, a denser fuel will provide more energy at the same load setting if all other factors are equal. Of course, fuel flow and engine performance are affected by other factors, such as fuel viscosity. Thus, all of the general relationships listed above hold between the reference diesel fuel and the methyl soyate and its blends with diesel fuel.

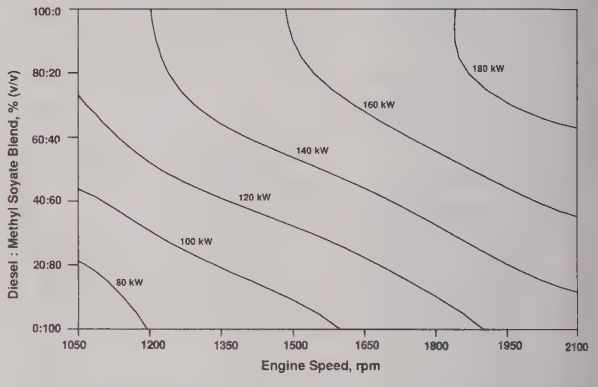

Fig. 1. Corrected power contouring of diesel:methyl soyate blends and engine speeds for Detroit diesel engine.

Engine performance

Detroit Diesel 6V-92

The corrected power outputs, at full load for all seven test fuels and eight speeds, are shown in Fig. 1. Statistical analyses conducted on effects of fuel blends and speeds on power output showed that there was a significant interaction between speeds and fuel blends ($P\gamma > F = 0{\cdot}0001$). Speed had a linear effect on the power output of the engine, while the diesel:methyl soyate blends had a quadratic effect. The maximum engine power developed with each fuel was obtained at 2100 rpm. The differences in power output at 2100 rpm between each fuel blend and neat diesel fuel are presented in Table 3. The engine power outputs on 90:10 and 80:20% (v/v) blends of diesel fuel:methyl soyate were increased by 4·06 and 1·79%, respectively, as compared to neat diesel fuel. The increase in power may have been due to the fact that when fuel was injec-

Table 3. Engine power and test fuel energy comparisons for Detroit diesel engine

	Diesel fuel (100%)	D+MS (90:10% v/v)	D+MS (80:20% v/v)	D+MS (70:30% v/v)	D+MS (50:50% v/v)	D+MS (25:75% v/v)	Methyl soyate (100%)
Power (kW) at full load and at 2100 rpm	189·5	197·2	192·9	186·3	167·9	154·5	132·5
Difference (%)	—	+4·06	+1·79	−1·69	−11·40	−18·47	−30·08
Brake specific fuel consumption at 2100 rpm (kg/kW-h)	0·3023	0·3007	0·3057	0·3116	0·338	0·3568	0·3664
Difference (%)	—	−0·53	+1·11	+3·06	+11·80	+18·01	+21·21
Gross heating value (mass basis)							
MJ/kg	45·5	44·78	44·31	42·93	41·95	40·98	40·07
Difference (%)	—	−1·58	−2·62	−5·65	−7·80	−9·93	−11·93
Gross heating value (volumetric basis)							
MJ/l at 15·6°C	38·14	37·84	37·35	36·26	36·04	35·80	35·55
Difference (%)	—	−0·79	−2·07	−4·93	−5·51	−6·14	−6·79
Heat supplied per unit fuel consumption							
MJ/kW-h	13·75	13·47	13·55	13·38	14·18	14·62	14·68
Difference (%)	—	−2·04	−1·45	−2·69	+3·12	+6·34	+6·78

D = Diesel fuel.
MS = Methyl soyate.

ted into a cylinder it heated up to its autoignition temperature before igniting. During this heating process, the fuel vaporized, removing heat from the intake charge. As more heat was removed from the intake charge the engine produced more power. There was a substantial decrease in power output on methyl soyate blends above 30%. The power loss was attributed to the lower energy content of the methyl soyate as compared to diesel fuel.

Assuming that the injection pump metered fuel equally on a volume basis, at the same load for all the test fuels, the differences between the gross heating values of the fuels and the differences in engine power output at the fuel load should be similar. However, this was not the case when these differences were compared. The power output also depended on the brake specific fuel consumption, discussed in the following section.

The torques produced by the engine at full load for all seven fuels and eight speeds are shown in Fig. 2. Statistical analyses conducted on effects of fuel blends and speeds on torque showed that there was a significant interaction between speeds and fuel blends ($F = 12{\cdot}20$, $P\gamma > F = 0{\cdot}0012$). Speed had a linear effect on the torque, while the diesel fuel:methyl soyate blends had a cubic effect. The maximum torque of 1122 Nm was developed at 1050 rpm with 100% diesel fuel. At constant speed, the torque was reduced to 1121 Nm with 90:10% (v/v), 1107 Nm with 80:20% (v/v) diesel fuel:methyl soyate blends. With the 70:30% (v/v) blend and lower diesel fuel concentrations there was a drastic reduction in torque at 1050 rpm. The minimum torque at this speed was 606 Nm with 100% methyl soyate.

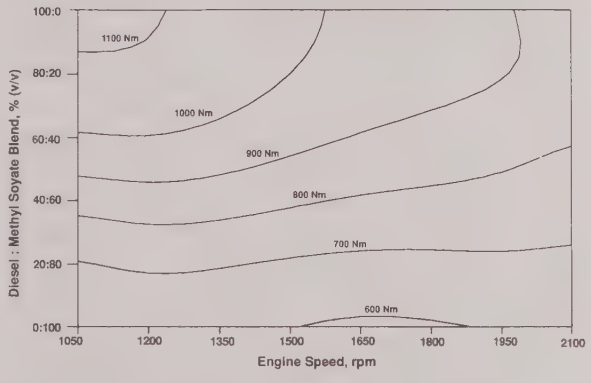

Fig. 2. Corrected torque contouring of diesel:methyl soyate blends and engine speeds for Detroit diesel engine.

Brake specific fuel consumption data at full load for all seven test fuels and eight speeds are shown in Fig. 3. There was no significant interaction between the fuel blends and speeds. However, there were significant main effects of fuel blend ($F = 744{\cdot}66$, $P\gamma > F = 0{\cdot}0001$) and speed ($F = 93{\cdot}33$, $P\gamma > F = 0{\cdot}0001$) on brake specific fuel consumption. Both were linear relationships. Fuel consumption was minimum at 1050 rpm for all test fuel blends. Similarly, fuel consumption reached a minimum with 100% diesel fuel and a maximum with 100% methyl soyate. Since the methyl soyate blended fuels were denser than the diesel fuel, and since the injection pump metered fuel on a volumetric basis, the mass fuel consumption increased with higher methyl soyate content blends. Thus, it makes more sense to compare fuel consumption on the basis of heating values. The heating values of the fuels should be

compared to determine the actual influence of the fuel on the engine power output. Table 3 shows that the gross heating value of the 90:10% (v/v) diesel fuel:methyl soyate blend was 0·79% less and the heat supplied per unit fuel consumption was reduced by 2·04% but the engine developed 4·06% more power than with 100% diesel fuel. Similarly, the heating value of the 80:20% (v/v) diesel fuel-:methyl soyate blend was 2·07% less and the heat supplied per unit fuel consumption was reduced by 1·45% but the engine developed 1·79% more power than with 100% diesel fuel. Thus, addition of methyl soyate to diesel fuel suggested an improvement in the combustion efficiency.

The heating values of diesel fuel:methyl soyate blends at 70:30% (v/v) and higher ratios were found to be reduced by 4·93% and more. For these blends, power output was reduced by 1·69% or more. At 100% methyl soyate fuel there was a 30% drop in power output at full speed. The Detroit Diesel 6-V 92 engine was electronically controlled to keep a set air–fuel ratio of about 40:1, so when fuel flow through the injectors was decreased, the electronic control compensated by lowering the air flow. Thus, the engine was operating as if it was at partial load and produced less power.

Cummins NTA-855-C

The corrected power output at full load for all four test fuels and seven speeds are shown in Fig. 4. Statistical analyses conducted on effects of fuel blends and speeds on power output showed that there was a significant interaction between speed and fuel blend ($F = 28·7$, $P\gamma > F = 0·0001$). Speed had a quadratic effect on the power output of the engine, while the diesel fuel:methyl soyate blend had a linear effect. The maximum engine power developed with each fuel was obtained at 2000 rpm. The differences in power output at 2000 rpm between each fuel blend and reference No. 2 diesel fuel are presented in Table 4.

Assuming that the injection pump metered fuel equally on a volume basis, at the same load for all the test fuels, the differences between the gross heating values of the fuels and the differences in engine power output at the full load should be similar. However, this was not the case when these differences were compared. The power output also depended on the brake specific fuel consumption, discussed in the following section.

The power developed on the first baseline was significantly better than with the second baseline ($F = 21·57$, $P\gamma > F = 0·0001$) for all fuel blends. That

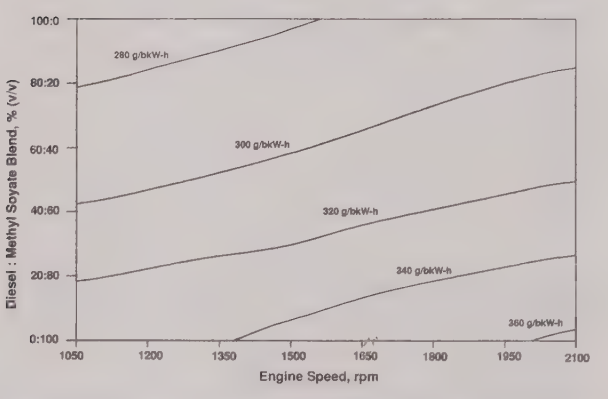

Fig. 3. BSFC contouring of diesel:methyl soyate blends and engine speeds for Detroit diesel engine.

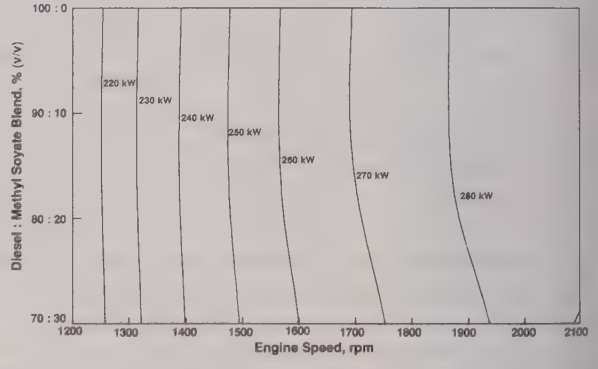

Fig. 4. Corrected power contouring of diesel:methyl soyate blends and engine speeds for Cummins diesel engine.

Table 4. Engine power and test fuel energy comparisons for Cummins engine

	Diesel fuel (100%)	Diesel fuel + methyl soyate (90:10% v/v)	Diesel fuel + methyl soyate (80:20% v/v)	Diesel fuel + methyl soyate (70:30% v/v)
Power (kW) at full load and at 2000 rpm (averaged over two base line)	286·7	286·85	286·35	282·75
Difference (%)	—	+0·05	−0·12	−1·38
Gross heating value of fuel (mass basis)				
MJ/kg	45·5	44·78	44·31	42·93
Difference (%)	—	−1·58	−2·62	−5·65
Gross heating value of fuel (volumetric basis)				
MJ/l at 15·6°C	38·14	37·84	37·35	36·26
Difference (%)	—	−0·79	−2·07	−4·93

showed that engine performance deteriorated with the type of fuel tested in the study.

The brake specific fuel consumptions at full load for all four test fuels and seven speeds are shown in Fig. 5. There was no significant interaction between the fuel blends and speeds. However, there were significant main effects of fuel blend ($F = 21{\cdot}76$, $P\gamma > F = 0{\cdot}0001$) and speeds ($F = 419{\cdot}25$, $P\gamma > F = 0{\cdot}0001$) on brake specific fuel consumption; both were quadratic relationships. Fuel consumption was minimum at speeds between 1600 and 1800 rpm for all test fuel blends. Similarly, fuel consumption was minimum with 90:10% (v/v) diesel fuel:methyl soyate blend. Fuel consumption was maximum with the 70:30% (v/v) blend. Since the methyl soyate fuels were denser than the diesel fuel, and since the injection pump metered fuel on a volumetric basis, the fuel consumption increased with higher methyl soyate content blends. Thus, it would make more sense to compare fuel consumption on the basis of heating values. The heating values of the fuels should be compared on a volume basis to determine the actual influence of the fuel's heating value on the engine power output tests. From Table 4 it is observed that the gross heating value of the 90:10% (v/v) diesel fuel:methyl soyate blend was 0·79% less and still developed 0·05% more power than with 100% diesel fuel. The heating values of 80:20 and 70:30% (v/v) diesel fuel:methyl soyate blends were found to be 2·07 and 4·93% less, respectively. For these two blends the power output was only 0·12 and 1·38% less, respectively, that suggested an improvement in the combustion efficiency of the fuels by addition of methyl soyate to diesel fuel.

Emissions analyses

Detroit Diesel 6V-92

The brake specific emissions for all the test fuels are shown in Figs 6–9. HC, NO_x, CO and Bosch smoke emissions were measured for the seven test fuel blends at eight engine speeds.

Hydrocarbon emission values for the seven test fuels at different speeds are shown in Fig. 6. Statistical analyses conducted for effects of test fuel blends and engine speeds on brake specific HC showed that there was a significant interaction between speed and test fuel blend ($F = 7{\cdot}86$, $P\gamma > F = 0{\cdot}0073$). As the engine speed increased there was a slight decrease in the HC emissions, but at a constant engine speed, as the methyl soyate content increased, there was a quadratic trend in the HC emissions. The HC emissions increased from an average of 0·4189 g/bkW-h (0·3123 g/bhp-h) at 100% diesel fuel to 0·7630 g/bkw-h (0·5689 g/bhp-h) for a blend of 70:30% (v/v) diesel fuel:methyl soyate. A

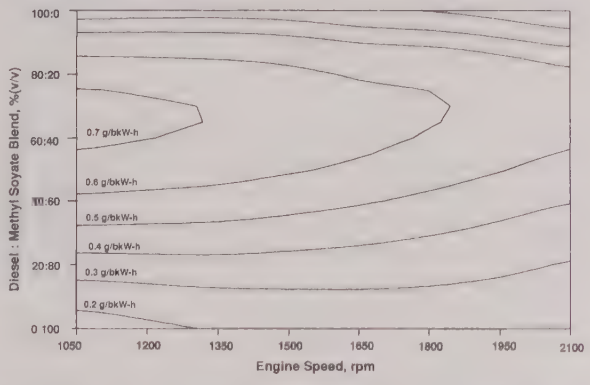

Fig. 6. BSHC emissions contouring of diesel:methyl soyate blends and engine speeds for Detroit diesel engine.

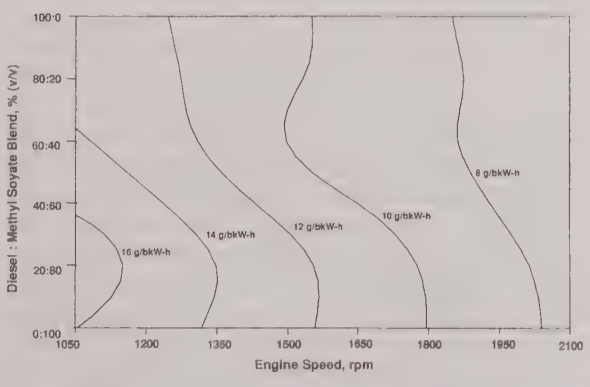

Fig. 7. $BSNO_x$ emissions contouring of diesel:methyl soyate blends and engine speeds for Detroit diesel engine.

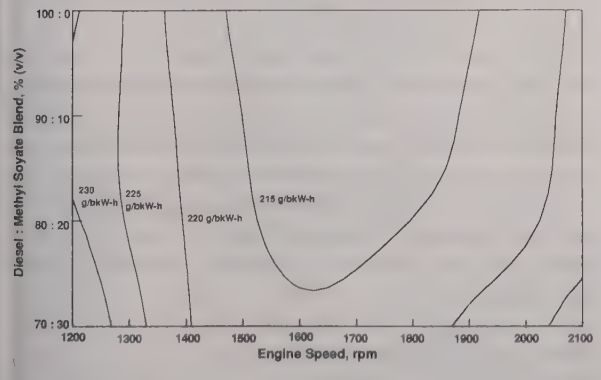

Fig. 5. BSFC contouring of diesel:methyl soyate blends and engine speeds for Cummins diesel engine.

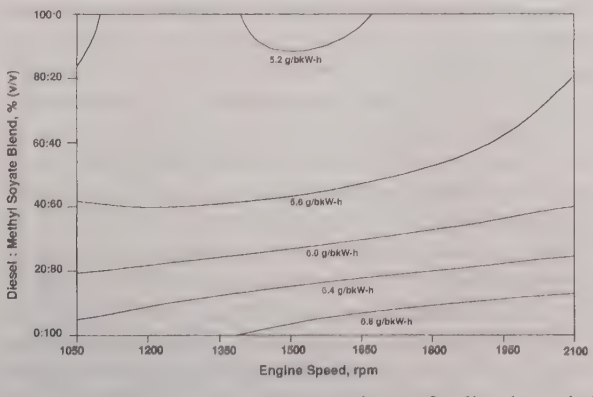

Fig. 8. BSCO emissions contouring of diesel:methyl soyate blends and engine speeds for Detroit diesel engine.

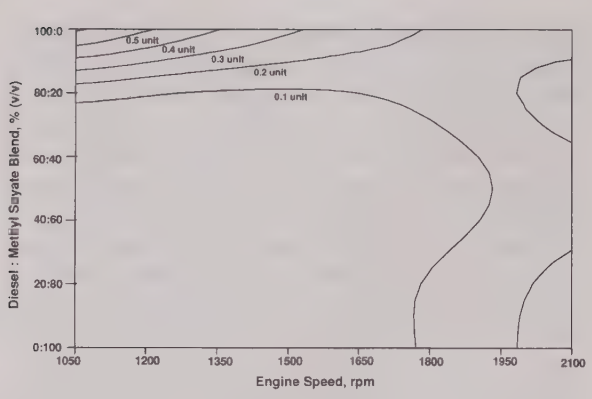

Fig. 9. BOSCH smoke emissions contouring of diesel:methyl soyate blends and engine speeds for Detroit diesel engine.

minimum of 0·1952 g/bkw-h (0·1455 g/bhp-h) HC emissions were observed for 100% methyl soyate.

Detroit Diesel 6V-92 engines are programmed to maintain air–fuel ratios of about 40:1, extremely lean even for diesel engines. The stoichiometric air–fuel ratio for diesel fuel is 14·4:1, and for oxygenated fuels this ratio is even less. For an oxygenated diesel fuel, the stoichiometric ratio is even leaner. The leaning effect, coupled with the undermixing of air and fuel due to larger droplet size (higher viscosity), accounted for the increase in the HC emissions produced by the methyl soyate blends of 20% or more.

While nitric oxide (NO) and nitrogen dioxide (NO_2) are usually grouped together as NO_x emissions, nitric oxide is the predominant oxide of nitrogen produced inside the engine cylinder. The principal source of NO is the oxidation of atmospheric nitrogen. The formation of NO_x depends on temperature. The effects of engine speeds and test fuel blends on NO_x emissions are shown in Fig. 7. Statistical analysis showed that there was a significant interaction between the engine speeds and test fuel blends ($F = 17·38$, $P\gamma > F = 0·0001$). Speed had a linear effect, while the test fuel blend has a cubic effect on NO_x emissions. NO_x emissions decreased with increasing engine speed. The average NO_x emissions were between 12 and 13 g/bkW-h (9 and 10 g/bhp-h) at 1050 rpm engine speed, and decreased to between 6·57 and 7·24 g/bkW-h (4·9 and 5·4 g/bhp-h) at a rated engine speed of 2100 rpm. At high diesel fuel contents, i.e. between 70 and 100%, the NO_x emissions remained fairly constant; but for lower diesel fuel content blends the NO_x emissions suddenly increased and then again decreased at 100% methyl soyate.

Carbon monoxide emission values for the seven fuel blends and eight engine speeds are shown in Fig. 8. Statistical analysis showed that there was a significant interaction between engine speeds and test fuel blends ($F = 6·53$, $P\gamma > F = 0·0139$). Brake specific CO (BSCO) emissions had a quadratic trend with speed. At an engine speed of 1650 rpm the BSCO emissions were minimum with 5·16 g/bkW-h (3·85 g/bhp-h) and increased to about 5·36 g/bkW-h (4 g/bhp-h) with either an increase or decrease in the engine speed. As diesel fuel content increased from 0 to 70% in the diesel fuel:methyl soyate blend, BSCO decreased from 7·38 g/bkW-h (5·5 g/bhp-h) to about 5·36 g/bkW-h (4 g/bhp-h) and then remained constant. This was a result of leaning of the air–fuel ratios relative to the stoichiometric air–fuel ratios when methyl soyate in the blend was increased. At higher diesel fuel contents (70% or more) the CO emissions remained more or less the same, between 5·23 and 5·5 g/bkW-h (3·9 and 4·1 g/bhp-h).

Bosch smoke units are an indication of particulate and soot formation in the exhaust of an engine. Particulates primarily contain carbon particles. From the increase in HC and CO emissions it would seem logical that smoke units should increase as well. The observed smoke readings, in Bosch smoke units, for test fuel blends at different speeds are shown in Fig. 9. A significant statistical interaction between speeds and test fuel blends was observed ($F = 13·45$, $P\gamma > F = 0·006$). The smoke had a quadratic trend with speed and a cubic trend with test fuel blend. From Fig. 7, although the trend of visible smoke with speeds and test fuel blends looks inconclusive, the general trend was that visible smoke was reduced with increasing speed and methyl soyate content in the fuel blend. More smoke was produced with 100% diesel fuel as compared to diesel fuel:methyl soyate blends at full load.

Not enough is known about the formation of soot and particulates; therefore, an explanation of the observed decrease would be hypothetical. Soot and particulates have two basic generation states: formation and growth. The formation stage usually involves dehydrogenation of unburned hydrocarbons, forming carbon nuclei for the particles. Then the growth stage occurs through coagulation and aggregation of other carbons in the exhaust. During formation and growth, oxidation of the particulate from an oxidizing species can occur producing CO and CO_2. One such oxidation species may exist in the combustion products of the methyl soyate, explaining the increase in CO emissions and the decrease in smoke units.

This theory is supported by the fact that soot oxidizes logarithmically based on the partial pressure of oxygen present in the exhaust. As the partial pressure of oxygen increases, soot oxidation increases, thereby reducing particulates. Methyl soyate is an oxygenated fuel. Therefore, the oxygen present in the fuel increases the partial pressure of oxygen present in the exhaust, thereby increasing soot oxidation.

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The brake specific emissions for all the test fuels are shown in Figs 10–13. HC, NO_x, CO and Bosch

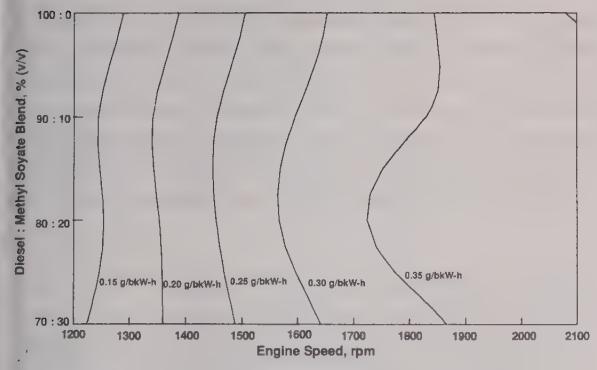

Fig. 10. BSHC emissions contouring of diesel:methyl soyate blends and engine speeds for Cummins diesel engine.

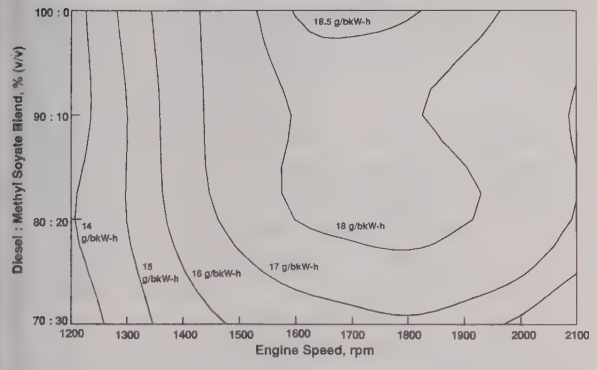

Fig. 11. $BSNO_x$ emissions contouring of diesel:methyl soyate blends and engine speeds for Cummins diesel engine.

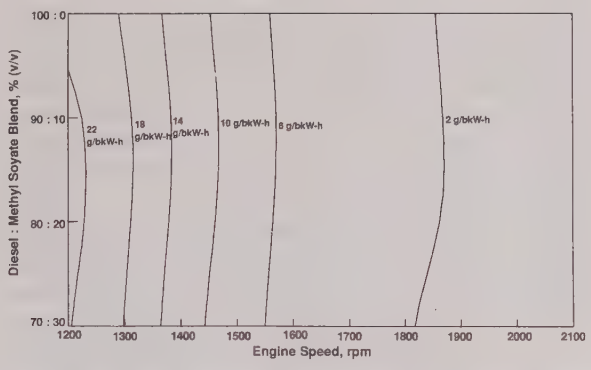

Fig. 12. BSCO emissions contouring of diesel:methyl soyate blends and engine speeds for Cummins diesel engine.

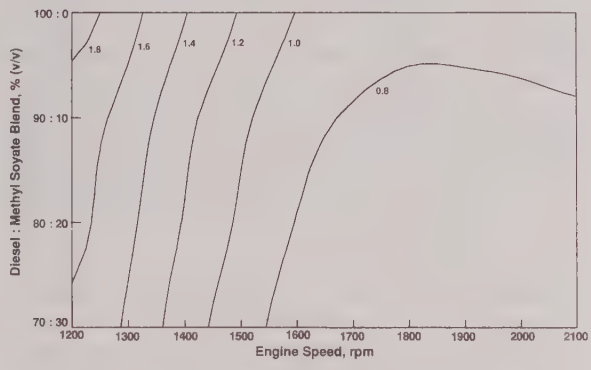

Fig. 13. BOSCH smoke emissions contouring of diesel:methyl soyate blends and engine speeds for Cummins diesel engine.

smoke emissions were measured for the four test fuel blends at seven engine speeds.

Hydrocarbon emission values for the four test fuels at different speeds are shown in Fig. 10. Statistical analyses conducted for effects of test fuel blends and engine speeds on brake specific HC showed that there was a significant interaction between speed and test fuel blend ($F = 6{\cdot}80$, $P\gamma > F = 0{\cdot}0135$). Both engine speed and diesel fuel:methyl soyate blends were found to have a quadratic effect on the hydrocarbon emissions. As the engine speed increased and diesel fuel content in the fuel blend increased there was an increase in the HC emissions. Maximum HC emissions were observed to be 0·416 g/bkW-h (0·31 g/bhp-h) for 100% diesel fuel at an engine speed of 2100 rpm. At constant speed, increasing the methyl soyate content in the blend resulted in reduced HC emissions. At 2100 rpm, the HC emissions for 100% diesel fuel reduced from 0·416 g/bkW-h (0·31 g/bhp-h) to 0·3619, 0·3843 and 0·3671 g/bkW-h (0·2698, 0·2865 and 0·2737 g/bhp-h) for the 90:10, 80:20 and 70:30% (v/v) blends, respectively. Similarly, as the engine speed decreased the HC emissions also decreased. The decrease in brake specific HC emissions with increasing methyl soyate content in fuel blends once again indicated improvement in the combustion efficiency of the fuels by addition of methyl soyate to diesel fuel.

In the case of the Detroit diesel engine there was a linear decrease in HC emissions with increasing engine speed and a quadratic trend in the HC emissions with an increase in the methyl soyate content in the fuel blends at the same speed. The two-stroke Detroit Diesel 6V-92 engine had an electronically controlled fuel injection system, whereas the four-stroke Cummins NTA-855-C engine had a mechanically controlled fuel injection system. In a two-stroke engine, since the power and exhaust strokes are combined, there will be loss of some of the unburned fuel, whereas in the case of a four-stroke engine the combustion can be completed even during the exhaust stroke. Therefore, the HC emissions with the four-stroke Cummins engine were less than the two-stroke Detroit diesel engine.

Effects of engine speeds and test fuels on NO_x emissions are shown in Fig. 11. Statistical analyses showed that the engine speeds and test fuel blends interaction had no significant effect on NO_x emissions. The main effects of speeds and fuel blend were significant. The cubic trend between test fuel blends and NO_x emissions was found to be signifi-

cant ($F = 26{\cdot}39$, $P\gamma > F = 0{\cdot}0001$). The quadratic trend between speeds and NO_x emissions was also significant ($F = 184{\cdot}06$, $P\gamma > F = 0{\cdot}0001$). NO_x emissions increased with increasing methyl soyate content in the blend. Minimum NO_x of 13·18 g/bkW-h (9·825 g/bhp-h) was observed with 100% diesel fuel at 1200 rpm engine speed. Also, NO_x emissions increased with increasing engine speed. Maximum NO_x was produced at engine speeds between 1800 and 2000 rpm at full load. At 1800 rpm, NO_x emissions were 16·57 g/bkW-h (12·355 g/bhp-h) with 100% diesel fuel and increased to 19·06, 17·68 and 18·80 g/bkW-h (14·21, 13·185 and 14·02 g/bhp-h) with 90:10, 80:20 and 70:30% (v/v) blends, respectively. Though the statistical analysis showed a cubic trend of NO_x emissions with blend, NO_x emissions data were still comparable to the reference diesel fuel.

With the Detroit Diesel 6V-92 engine a decrease in NO_x emissions with increasing speed was observed. The maximum NO_x emissions with 70:30% (v/v) diesel:methyl soyate blend and above in the Detroit diesel engine were 13·5 g/bkW-h (10 g/bhp-h), whereas in the Cummins engine the minimum emissions were around 13·5 g/bkW-h (10 g/bhp-h).

Wagner *et al.* (1984) reported a two- to five-fold increase in NO_x emissions when they compared 100% methyl, ethyl and butyl esters of soybean oil in an engine at 100% load. The exhaust gas temperature in their study was 20–70°C higher with ester fuels as compared to a reference diesel fuel. In the present study there was no difference in the exhaust gas temperatures for the fuel blends.

Carbon monoxide emission values for the four fuel blends and seven engine speeds are shown in Fig. 12. Statistical analysis once again showed that there was no significant interaction between engine speeds and test fuel blends. The main effects of speed and fuel blend were significant. Brake specific CO emissions had a quadratic trend with fuel blend ($F = 7{\cdot}66$, $P\gamma > F = 0{\cdot}0087$) and a cubic trend with speeds ($F = 4{\cdot}36$, $P\gamma > F = 0{\cdot}0436$). It was observed from Fig. 4 that brake specific CO emissions were very similar for all four fuel blends at speeds from 1200 to 2100 rpm. At lower engine speeds the CO emissions were 10–15 times higher than at higher engine speeds.

Carbon monoxide emissions for the first baseline were significantly lower than for the second baseline ($F = 76{\cdot}84$, $P\gamma > F = 0{\cdot}0001$) at all fuel blends and speeds. Once again, this shows that the performance of the engine deteriorated as the methyl soyate content in the blend increased.

The observed smoke readings, in Bosch smoke units, for diesel fuel:methyl soyate blends at different speeds are shown in Fig. 13. No significant statistical interactions between speeds and test fuel blends were observed. However, a cubic trend with blend ($F = 13{\cdot}60$, $P\gamma > F = 0{\cdot}0007$) and a quadratic trend with engine speeds ($F = 324{\cdot}89$, $P\gamma > F = 0{\cdot}0001$) were found to be statistically significant. Visible smoke was reduced with increasing speeds and also with increasing methyl soyate contents in the blends. More smoke was produced with neat diesel fuel as compared to diesel fuel:methyl soyate blends at full load. A similar trend was observed with the Detroit diesel engine.

CONCLUSIONS

1. The neat ester had high density and viscosity and low API gravity and heating values as compared to No. 2 diesel fuel.
2. Methyl soyate blended with diesel fuel had fuel properties comparable to diesel fuel.
3. Engine performance with diesel fuel:methyl soyate blends did not differ to a great extent up to a 70:30% (v/v) from that of diesel-fueled engine performance. In fact, there was an increase in power output by blending methyl soyate with diesel fuel up to 80:20% (v/v) as compared to neat diesel fuel with no increase in fuel consumption.
4. Reduction in power output was less than the corresponding reduction in heating value of methyl soyate, indicating more complete combustion of methyl soyate fuels as compared to neat diesel fuel.
5. Hydrocarbon emissions were not affected by speed of the engine but had a quadratic trend with the diesel fuel:methyl soyate blend with the Detroit diesel engine whereas, in case of the Cummins engine it decreased with an increase in methyl soyate content in the diesel:methyl soyate blend indicating an improvement in the combustion efficiency of the fuel.
6. There was slight increase in NO_x emissions with increasing methyl soyate content in the blend at higher speeds but at lower speeds there was a quadratic trend with diesel fuel content.
7. Carbon monoxide emissions were very similar for blends up to 70:30% (v/v) diesel fuel:methyl soyate blends at any one speed. However, both engines performed differently for CO emissions.
8. The effects of engine speed and diesel fuel:methyl soyate blend on Bosch smoke was inconclusive. In general, visible smoke decreased with increasing speed and methyl soyate content. More smoke was produced with neat diesel fuel at full load.

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MICROBIAL PROTEIN PRODUCTION BY *PAECILOMYCES VARIOTII* CULTIVATED IN EUCALYPTUS HEMICELLULOSIC HYDROLYZATE

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Abstract

The eucalyptus hemicellulose fraction was hydrolyzed by treating eucalyptus wood chips with 1·2% (w/v) sulfuric acid (wood to acid ratio of 1:3) at 150°C for 120 min. The hydrolyzate was used as substrate to grow Paecilomyces variotii *IOC-3764 in a 7-l fermenter at 30°C; air 1·5 vvm; stirring 400 rpm. The kinetic parameters measured included maximum growth rate (μ_{max} 0·10/h), yield ($Y_{x/s}$ 0·44 g/g), and productivity (Q_x 0·26 g/l·h). The protein produced (34% of the biomass) contained all essential amino acids for animal feed.*

Key words: Fermentation, xylose, glucose, acetic acid, furfural, *P. variotii*.

INTRODUCTION

Many lignocellulose materials have been studied as substrates for the production of microbial protein. Agro-industrial residues such as sugarcane bagasse (Pessoa Jr, 1991), rice straw (Almeida, 1991), corncobs (Gonzalex-Valdes & Moo-Young, 1981) and eucalyptus (Silva, 1991; Almeida e Silva *et al.*, 1995), which contain about 30% hemicellulose, have been hydrolyzed to produce a solution rich in xylose. Other sugar-rich industrial byproducts, such as vinasse, spent sulfite liquor and hemicellulose rayon hydrolyzates have also been tried as fermentation media (Bajpai & Bajpai, 1987; Lo & Moreau, 1986; Kiyan, 1988). Abu-Ruwaida *et al.* (1990) used a less common substrate, methanol.

Paecilomyces variotii, a fungus frequently found in air and soil in tropical areas, has been utilized for the production of microbial protein because of its excellent ability to grow in a variety of highly-polluting industrial effluents, such as molasses, wood hydrolyzates, spent sulfite liquor and vinasse (Romantschuk & Lehtomaki, 1978; Cabib *et al.*, 1983; Castlla *et al.*, 1984; Bajpai & Bajpai, 1987). *Paecilomyces variotii* was the first fungus to be utilized in an industrial process for the production of microbial protein. The process, known as 'Pekilo', is a continuous process in which the fungus is grown in sulfite liquor with a production of 10000 ton/year (Romantschuk & Lehtomaki, 1987). Brazil is ranked fourth in the world concerning homogeneous forest areas. It has 5·5 million hectares planted with eucalyptus, with an annual increment of 400 thousand hectares. Also, its productivity is among the highest in the world with 100 m^3 per hectare per year (Afonso Neto, 1986; Paper & Cellulose, 1988). Brito *et al.* (1979) showed that only 51·6% of the total dry mass of eucalyptus is utilized by the Brazilian industry. The rest of this biomass (branches, leaves, small trees, etc.) is left on the fields. Eucalyptus wood contains 40–62% cellulose, 12–22% hemicellulose, and 15–22% lignin (Vital & Della Lucia, 1986). The hemicellulose fraction can be easily removed by acid treatment and the resulting hydrolyzate is rich in fermentable sugars, mainly xylose (Dale, 1987).

The objective of the present study was to evaluate the eucalyptus hemicellulose hydrolyzate as a fermentation medium for the production of microbial protein by *Paecilomyces variotii* as part of an effort to better utilize and also dispose of this abundant agro-industrial residue.

METHODS

Preparation and characterization of the hydrolyzate

Eucalyptus chips from the species *Eucalyptus grandis* (average size of $20 \times 10 \times 5$ mm with 30% moisture) were submitted to acid hydrolysis under the following conditions: wood chip mass, 200 kg; volume of acid solution to wood mass ratio, 3 (l/kg); tempera-

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ture, 150°C; reaction time, 120 min; concentration of sulfuric acid, 1·2% (w/v). Total reducing sugars (TRS), acetic acid, furfural and pH of the resulting hydrolyzate were determined.

Microorganism and cultivation

Paecilomyces variotii IOC-3764 was kept on malt extract agar slants at 4°C. The inoculum for the 7-l fermenter was prepared from a suspension of non-germinated spores cultivated in 250-ml Erlenmeyer flasks with 50 ml of malt extract agar at 30°C for 96 h. The hydrolyzate was submitted to treatment with flowing steam for 20 min. A volume of 2·5 l of this hydrolyzate was transferred to a 7-l fermenter (New Brunswick, model CF 500). Urea and rice-bran solutions sterilized at 121°C for 20 min were added to the hydrolyzate under aseptic conditions to produce final concentrations of these nutrients of 1 and 40 g/l, respectively. The inoculum used consisted of a 50 ml aliquot of the spore suspension described above at a concentration of 10^6; aeration 1·5 vvm; agitation 400 rpm; time 72 h. Aliquots of 20 ml were taken at different times for the determination of pH and furfural, acetic acid, total reducing sugar (TRS) and dry mass content. All experiments were made in duplicate, except for the determination of partial composition of the eucalyptus wood hydrolyzate that was made in triplicate. Average values are reported throughout the manuscript.

Analytical methods

Total reducing sugars (TRS) were determined as glucose by the Somogyi–Nelson method according to Saeman *et al.* (1945). Glucose, xylose and acetic acid were determined by HPLC (HPX-87H Bio-Rad column with a RI 16X detector): aliquots of 20 μl were analyzed at 45°C with 0·01 N sulfuric acid as the eluent (flow rate of 0·6 ml/min). Furfural was analyzed also by HPLC (20 μl of sample injected) under the following conditions: RP18HP column, acetic acid:acetonitrile:water solution (1:10:80 volume ratio) as eluent with a flow rate of 0·8 ml/min, temperature of 25°C, UV detector.

The biomass in the fermenter was determined gravimetrically after vacuum filtration of an aliquot of fermented medium. The mycelium was retained as a cake that was washed twice and dried at 80°C. Total protein was determined by a semi-micro Kjeldahl method according to Silva (1990). Amino acid content was determined by the method of Araujo and D'Souza (1986) using an Aminocron II amino acid analyzer.

Fermentation kinetic parameters

The maximum specific growth rate (μ_{max}) was determined from the growth curve in the exponential phase according to Le Duy and Zajic (1973). The yield ($Y_{x/s}$) was calculated as grams of dry cell produced per gram of reducing sugars used. The productivity (gcell/g.h) was determined by the ratio of cell concentration variation and sugar concentration in a defined fermentation time (72 h).

RESULTS AND DISCUSSION

The results of a partial characterization of the hemicellulose acid hydrolyzate of eucalyptus chips are presented in Table 1. Besides the expected high concentration of sugars in the hydrolyzate, there was also a high concentration of acetic acid and furfural. These two compounds are known to be present in such hydrolyzates and to act as inhibitors of microbial growth (Tran & Chambers, 1985).

The batch fermentation of this hydrolyzate with *Paecilomyces variotii* (Fig. 1) was characterized by lag and exponential phases of 30 and 24 h, respectively. The beginning of microorganism growth was observed at a furfural concentration of about 1 g/l. Biomass production corresponded to 6·35 g/l with a maximum specific growth rate (μ_{max}) of 0·10/h, yield ($Y_{x/s}$) of 0·44 g/g, and productivity (Q_x) of 0·26 g/l.h.

Furfural was present in the medium at initial concentration of 2·4 g/l and decreased continuously until total depletion at 36 h of fermentation. Furfural was probably converted to furfurilic alcohol by

Table 1. Partial composition of the eucalyptus wood hydrolyzate

pH	1·82
Dry mass (g/l)	38·9
TRS (g/l)	29·1
Xylose (g/l)	14·6
Glucose (g/l)	10·2
Furfural (g/l)	5·0
Acetic acid (g/l)	6·8

Average triplicate values.
TRS: Total reducing sugar.

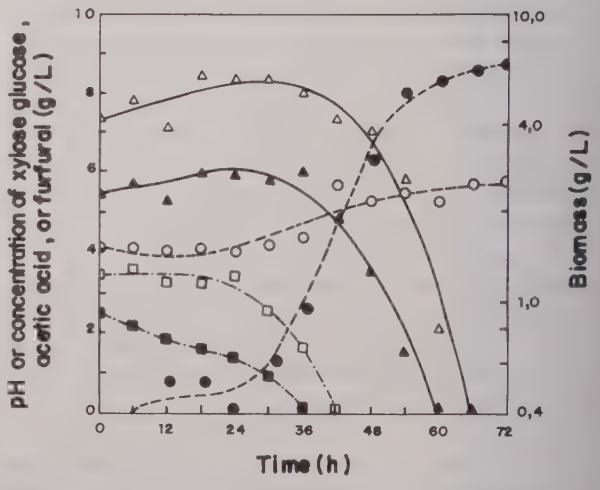

Fig. 1. *Paecilomyces variotii* growth in eucalyptus hemicellulose hydrolyzate. Biomass production (——●——), pH (——○——), concentrations of xylose (——△——), glucose (——▲——), acetic acid (——□——), and furfural (——■——).

Table 2. Comparison of the amino acid profile of the protein produced by *Paecilomyces variotii* grown in eucalyptus wood hydrolyzate with that of other proteins

Amino acid	Concentration (as percentage of total protein) *Paecilomyces variotii*				
	IOC-3764[a]	Pekilo[b]	FAO[c]	Soybean[c]	Animal feed[d]
Alanine	7·19	5·80	—	—	—
Valine	6·39	5·03	4·20	5·00	2·70
Glycine	4·99	4·77	—	—	2·43
Isoleucine	5·08	4·18	4·20	4·90	2·57
Leucine	14·38	6·99	4·80	8·00	3·80
Proline	5·98	4·23	—	—	—
Threonine	4·65	4·25	2·80	4·30	1·97
Serine	3·39	4·91	—	—	—
Methionine	1·74	1·73	2·20	1·30	0·72
Phenylalanine	4·31	3·80	2·80	5·30	2·20
Aspartic acid	7·03	8·12	—	—	—
Glutamic acid	11·41	10·35	—	—	—
Tyrosine	5·86	3·36	—	—	—
Lysine	7·34	5·60	4·20	6·60	3·20
Arginine	4·31	6·02	—	—	—
Histidine	2·18	2·15	—	—	—
Cystine	1·17	1·38	2·00	1·60	0·74
Tryptophan	—	—	1·40	1·40	0·60

[a]Values are means of duplicates.
[b]Farstad *et al.* (1975).
[c]Araujo & D'Souza (1986).
[d]Lo & Moreau (1986).

the fungus, as observed in yeast by Weigert *et al.* (1988). Acetic acid was also totally depleted from the medium after 42 h of fermentation, leading to a slight increase in the fermentation broth pH. A similar phenomenon was observed by Cabib *et al.* (1983) for the growth of *Paecilomyces variotii* in vinasse.

An increase in glucose and xylose concentrations was observed during the first 36 h of fermentation, probably due to liquid loss by aeration and agitation, as also observed by Doin (1976). After this period, these sugars were continuously consumed, being depleted from the medium after 60 and 66 h of cultivation, respectively.

The biomass produced had a total protein content of 34%. Its amino acid composition is shown in Table 2, together with the amino acid profile of three animal feed protein standards. The amino acid profile of the protein from *Paecilomyces variotii* growth in the eucalyptus hydrolyzate was similar to the profile of the protein obtained by the 'Pekilo' process, except for leucine content, which was double the value for the 'Pekilo' process. When compared to the FAO and soybean protein standards, one can see that the protein produced by *Paecilomyces variotii* cultivated in eucalyptus hemicellulose hydrolyzate contains all the essential amino acids for animal feed. Moreover, it has a superior profile when compared with the plant protein.

The present results demonstrate the potential of eucalyptus hemicellulose hydrolyzate as a substrate for the production of microbial protein by the cultivation of *Paecilomyces variotii* IOC-3764. Eucalyptus hemicellulose can be efficiently hydrolyzed by a simple method and the sugar solution obtained can be used as a fermentation medium. The quality of the protein is equal to, or may exceed, that of conventional products in terms of its amino acid profile and the standards set for animal feed.

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